

AD _____

Award Number: W81XWH-06-1-0148

TITLE: Enhancing Tumor Drug Delivery by Laser-Activated Vascular Barrier Disruption

PRINCIPAL INVESTIGATOR: Bin Chen, Ph.D.
Chong He, Ph.D.

CONTRACTING ORGANIZATION: University of the Sciences in Philadelphia
Philadelphia, PA 19104

REPORT DATE: December 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-12-2006		2. REPORT TYPE Annual		3. DATES COVERED 30 Nov 2005 – 29 Nov 2006	
4. TITLE AND SUBTITLE Enhancing Tumor Drug Delivery by Laser-Activated Vascular Barrier Disruption				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0148	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Bin Chen, Ph.D., Chong He, Ph.D. Email: b.chen@usip.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of the Sciences in Philadelphia Philadelphia, PA 19104				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT An obstacle for successful drug therapy for cancer is the existence of drug delivery barriers, which causes insufficient drug delivery to the tumor tissue. Because of inadequate drug delivery to the tumor tissue, the drug dose has to be increased, which leads to normal tissue toxicity. This delivery problem not only limits the clinical application of existing chemotherapeutics, but also decreases the effectiveness of many new drugs under development for prostate cancer. We found that vascular targeting photodynamic therapy (PDT), a modality involving the combination of a photosensitizer and laser light, is able to disrupt tumor vascular barrier, a significant hindrance to drug delivery. Therefore, tumor accumulation of circulating molecules is significantly enhanced, as demonstrated by intravital fluorescence microscopy and whole-body fluorescence imaging techniques. Immunofluorescence staining of endothelial cytoskeleton structure further indicates microtubule depolymerization, stress actin fiber formation and intercellular gap formation. Based on these results, we propose to use this laser-based therapy to enhance anticancer drug effectiveness. PDT is currently in worldwide multicenter clinical trials for the localized prostate cancer therapy. The available results indicate that PDT employing advanced laser fiber technology and sophisticated light dosimetry is able to treat localized prostate cancer in an effective and safe way. The combination of photosensitization with current chemotherapy or other new drug therapies will greatly improve clinical treatment for localized prostate cancer patients that accounts for more than 90% of total prostate cancer population.					
15. SUBJECT TERMS Photodynamic therapy, verteporfin, tumor vascular targeting, vascular permeability, imaging					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	42	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusions.....	8
References.....	N/A
Appendices.....	8

Introduction

An obstacle to successful cancer drug therapy is the existence of drug delivery barriers, which results in insufficient and heterogeneous drug delivery to the tumor tissue. This drug delivery problem not only limits the clinical application of existing chemotherapeutics, but also decreases the effectiveness of many new drugs under development. Photodynamic therapy (PDT), a modality involving the combination of a photosensitizer and laser light, is an established cancer therapy. Over the past years, we have been focusing on developing PDT as a modality for tumor vascular targeting. Our recent results demonstrate that vascular-targeting PDT can be not only used to eradicate tumor tissue, but also to modify vascular barrier function for an enhanced drug delivery. This project will study in detail how vascular photosensitization permeabilizes blood vessels and the effects of photodynamic vascular targeting on tumor vascular function and drug delivery. We will use several fluorescence imaging systems in our study. These imaging modalities include both dynamic live animal/cell imaging that is capable of providing longitudinal information in real time and static ex vivo imaging that is able to reveal biological details at high resolution.

Body

Task 1. To investigate the molecular mechanisms by which photosensitization disrupts endothelial barrier function (months 1-12).

(a). Assess the correlation between photosensitization-induced microtubule disassembly and increase in endothelial cell permeability. The purpose of this study is to determine the role of microtubules in photosensitization-induced endothelial barrier function alteration (months 1-4).

We have found that microtubules play an important role in photosensitization-induced endothelial morphological and functional changes. As shown in the appended Clinical Cancer Research paper, microtubule depolymerization was noted shortly (5 min) after photosensitization treatment. Following microtubule depolymerization, we found stress fiber actin formation and consequent endothelial morphological changes. Endothelial barrier function assay also demonstrates that photosensitization is able to induce dose-dependent vascular barrier disruption. Taken together, our data suggest that photosensitization induces microtubule disassembly, which triggers endothelial cell morphological changes and barrier dysfunction.

(b). Elucidate the mechanism by which photosensitization-induced microtubule depolymerization triggers endothelial cell morphological and functional changes. In particular, we will examine whether Rho activation is involved and the downstream Rho/Rho kinase signaling pathway is functional in this process (months 5-12).

We have examined Rho activity after photosensitization treatment in the SVEC-4 endothelial cells. But our results are not consistent and we are not sure whether Rho/Rho kinase pathway is active after PDT. This is because Rho is a small molecule protein (~20 kDa) and only a small percentage of Rho protein is activated in response to various stimuli (usually less than 5%). We have to use a special pulldown method to detect this active form of Rho protein. We are repeating this experiment by further optimizing our experimental conditions.

We are also determining other signaling pathways that are activated by PDT to induce endothelial cell morphological changes. As shown in **Fig 1**, myosin light chain kinase (MLCK) is clearly up-regulated. MLCK is an important protein in regulating endothelial contraction. MLCK activation will lead to MLC phosphorylation, which induces cell contraction. We are currently determining the phosphorylation status of MLC.

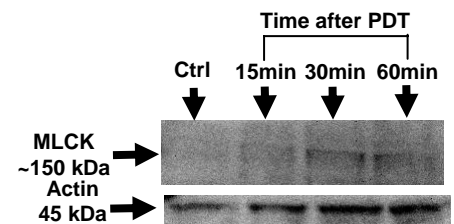


Fig. 1. Western blots of MLCK after photosensitization treatment with verteporfin. The SVEC-4 endothelial cells were treated with 5 mW/cm² light for 100 sec after incubation with 200 ng/ml verteporfin for 15 min. Cell lysates were prepared at different time points after treatment.

Task 2. To determine the functional change and the structural basis of photosensitization-induced vascular barrier compromise (months 1-24)

(a). Intravital microscopic study of photosensitization-induced vascular functional changes. Determine tumor hemodynamics, vascular permeability and vessel pore cutoff size changes in the orthotopic MatLyLu prostate tumors after varied doses of photosensitization treatment with verteporfin.

To determine tumor vascular functional changes in real time and at a high resolution, we used intravital fluorescence microscopy, which is able to continuously image both blood vessel morphological and functional changes after vascular-targeting PDT in live animals. To visualize functional blood vessels, we injected FITC-dextran with a molecular weight of 2000 kDa. Since this probe is a macromolecule, the extravasation is limited even in tumor blood vessels. **Fig 2** shows the intravital microscopic images right before, during and after vascular-targeting PDT. PDT clearly increases vascular permeability during and after PDT treatment, as indicated by the increase of FITC fluorescence intensity (**Fig 2B**). Vessel constriction is another significant event following vascular-targeting PDT. Vessel diameter changes are shown in **Fig 2A, 2C**. It can be seen that vessel constriction is especially significant in small blood vessels (generally less than 40 μm). Some small blood vessels were so constricted that they were barely visible after PDT.

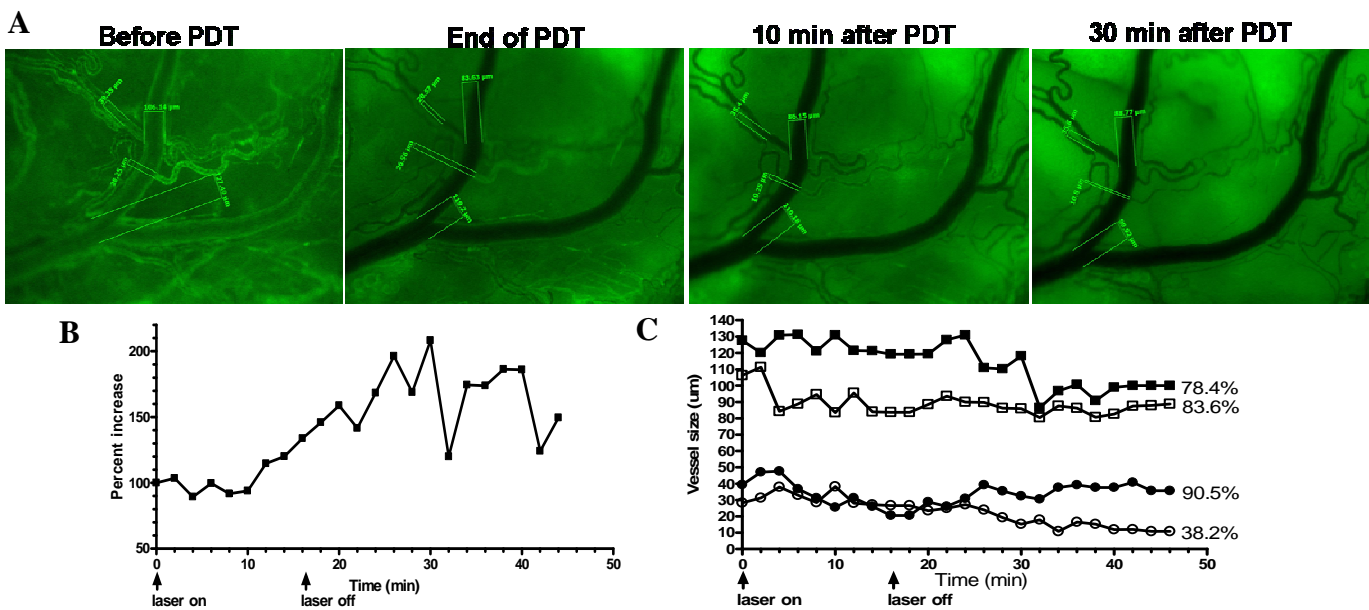


Fig. 2. Intravital fluorescence microscopic imaging of orthotopic MatLyLu rat prostate tumors treated with vascular-targeting PDT (50 J/cm^2 at 15 min after 0.25 mg/kg verteporfin (i.v.)). To visualize blood vessels, tumor-bearing animals were i.v. injected with 10 mg/kg 2000 kDa FITC-dextran before treatment. Tumors were imaged right before PDT and then every 2 min until 30 min after PDT. (A) Images showing before PDT, immediately end of PDT, 10 min and 30 min after PDT. (B) Changes in fluorescence intensity of each image (indicative of vascular permeability) relative to before PDT image during and after PDT. (C) Changes in blood vessel size during and after PDT. Numbers show the percentage change as compared to the pretreatment values.

We also i.v. injected Hoechst dye to the animals to label the blood cells and quantify blood flow rate. Although we have not finished analyzing all the images, we noticed that photosensitization-induced hemodynamic changes are highly heterogeneous and dependent upon the type of vessels, vessel diameter and pretreatment blood flow. Hemodynamic data will be included in the next report.

(b). Assessment of tumor uptake of fluorescence probes with different sizes. This experiment will allow us to evaluate the effect of tumor vascular permeabilization on the delivery of various fluorescence probes with similar sizes to chemotherapeutic agents, antibodies, nanomaterials and gene vectors.

Vascular functional changes will likely affect tumor uptake of circulating agents. We have measured tumor uptake of albumin-Evans blue and 2000 kDa FITC-dextran in the MatLyLu rat prostate tumor (please refer to the appended Clinical Cancer Research paper). Our results demonstrate that photosensitization is able to enhance tumor uptake of these different molecular weight macromolecules.

Tumor uptake of fluorescence probes can also be easily monitored in real time on live animals with whole body fluorescence imaging system. As shown in **Fig 3**, the vascular leakage of fluorescence-labeled albumin (TRITC-albumin) is significantly increased after the vascular-targeting PDT, as compared to the control tumor. This increase in vascular permeability appeared to be dependent upon the PDT dose. Increase in light dose was able to induce more leakage of albumin. Interestingly, PDT-induced increase in TRITC-albumin accumulation was especially pronounced in the tumor periphery. To further confirm these macroscopic imaging results, we sacrificed animals at various time points and excised tumor tissues for fluorescence microscopic study. **Fig 4** shows the TRITC fluorescence images of frozen tumor sections. Similar to the whole body tumor images, TRITC-albumin was found to have more accumulation in the tumor periphery.

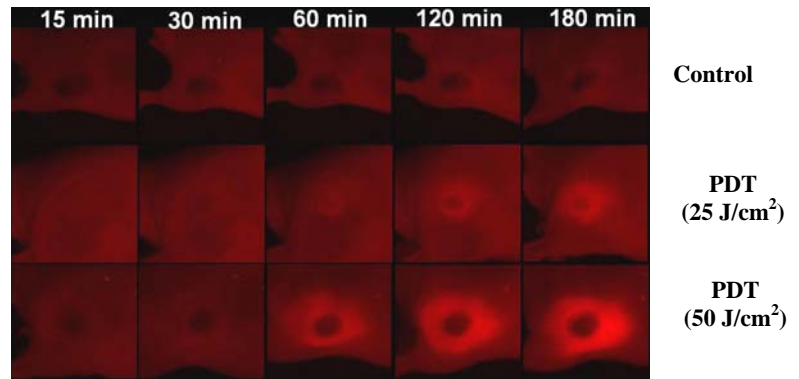


Fig.3. Whole body fluorescence imaging of increased vascular leakage of TRITC-albumin after photodynamic vascular targeting. The MatLyLu prostate tumors in athymic nude mice were treated with 25 J/cm² (middle panel) or 50 J/cm² (bottom panel) at 15 min after 0.25 mg/kg verteporfin (i.v.). Immediately after PDT, animals were i.v. injected with 20 mg/kg TRITC-albumin and imaged at various time points. Top panel: control tumor with 0.25 mg/kg verteporfin only.

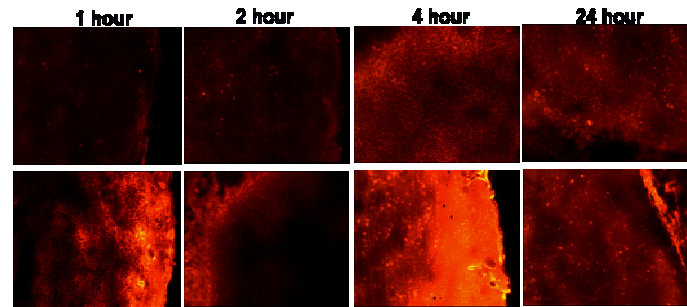


Fig.4. Fluorescence microscopic imaging of TRITC-albumin accumulation in the MatLyLu tumor periphery after vascular-targeting PDT. The MatLyLu tumors in athymic nude mice were treated with 50 J/cm² (bottom panel) at 15 min after 0.25 mg/kg verteporfin (i.v.). Immediately after PDT, animals were i.v. injected with 20 mg/kg TRITC-albumin. Tumors were sectioned at various time points after injection and imaged for TRITC-albumin fluorescence. Top panel: control tumor without PDT treatment. All images were captured to show tumor periphery

(c). Determine blood vessel structural changes induced by photosensitized vascular permeabilization. Light and electron microscopy and immunohistochemistry technique will be used to examine vessel structural alterations. In progress.

Task 3. To explore the potential of improving tumor drug delivery and therapeutic effect by photosensitized vascular permeabilization (months 25-36).

a. Fluorescence imaging, microscopy & flow cytometry analysis of tumor drug distribution and penetration. Uptake of chemotherapeutic drug mitoxantrone and antibody MDX-H210 (anti-HER2 x CD64) will be quantified by non-invasive whole body fluorescence imaging system, fluorescence microscopy and flow cytometry. Limitation of anticancer drug delivery and enhancement by photosensitized vascular permeabilization will be assessed at whole body, tumor tissue and tumor cell levels.

Antibody MDX-H210 is no longer available for research as the company has discontinued this product. Instead, we have chosen bevacizumab (Avastin) in this project. Bevacizumab is a FDA-approved recombinant humanized monoclonal antibody (MW 149 kDa) that binds to VEGF. We have labeled bevacizumab with Alex Fluor 647 dye using Invitrogen small animal in vivo imaging protein labeling reagents and are going to examine the influence of verteporfin-PDT on the distribution of bevacizumab.

b. Evaluate tumor response following the combination of anticancer agents (mitoxantrone or MDX-H210) and verteporfin-photosensitization. This study intends to demonstrate that photosensitized vascular permeabilization will lead to a more effective and safer use of conventional chemotherapeutics and new anticancer agent.

We have started to evaluate tumor response following the combination therapy. This is a key experiment for the whole project. We are glad to see that this on-going experiment has revealed promising results. We combined verteporfin-mediated photodynamic therapy with antibody drug bevacizumab in the PC3 human prostate tumor model. As shown in **Fig 5**, the average tumor volume in the group of animals treated with the combination therapy is only about half of the PDT alone group.

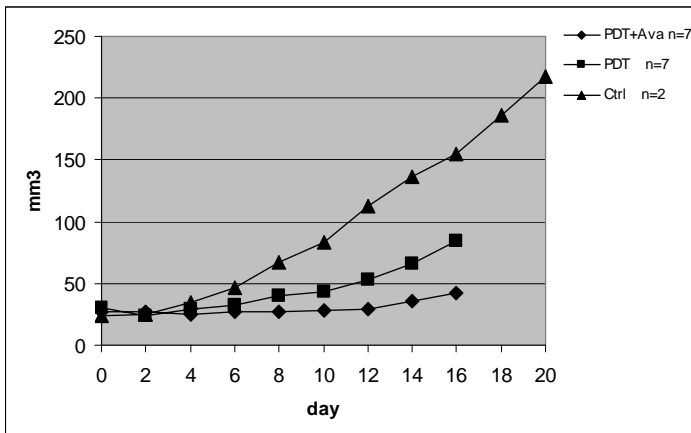


Fig. 5. Tumor regrowth curve after different treatments. For the PDT only group, PC3 human prostate tumors were treated with vascular-targeting PDT (40 J/cm² at 15 min after 0.5 mg/kg verteporfin (i.v.)). For the PDT + Ava group, animals were injected with 50 mg/kg Avastin (bevacizumab) immediately after PDT treatment. The control group received no treatment.

Key research accomplishments

- Vascular-targeting PDT induces significant morphological and functional changes in tumor blood vessels. It causes vessel constriction and vascular barrier dysfunction.
- PDT-induced vascular barrier dysfunction leads to increased accumulation of circulating macromolecules in tumor tissues, which can be used to enhance drug delivery to the tumor tissue. This effect appears especially significant in peripheral tumor area.
- Photosensitization induces microtubule depolymerization and stress fiber actin formation, leading to endothelial morphological changes. MLCK activation is involved in this process.
- Combination of vascular-targeting PDT and antibody drug bevacizumab (Avastin) results in an enhanced anti-tumor effect.

Reportable outcomes

Publications:

Chen B, Pogue BW, Luna J, Hardman R, Hoopes PJ, Hasan T. Tumor vascular permeabilization by vascular-targeting photosensitization: effects, mechanism and therapeutic implications. *Clin Cancer Res.* 2006, 10: 917-23.

Chen B, Pogue BW, Hoopes PJ, Hasan T. Vascular and cellular targeting for photodynamic therapy. *Crit Rev Eukaryot Gene Expr.* 2006, 16: 279-306.

Abstracts:

Chen B, He C, Crane C, Agharkar P, Pogue BW. Fluorescence imaging of verteporfin-mediated photodynamic therapy targeting prostate tumor vasculature. *Program & Abstracts of 11th World Congress of the International Photodynamic Association (IPA, March 28-31, 2007, Shanghai, China).*

Chen B, Pogue BW, Hoopes PJ, Hasan T. Effects and mechanisms of vascular permeabilization by vascular-targeting photodynamic therapy. *Conference Proceedings of the 33rd Meeting of the American Society for Photobiology (Jul 8-12, 2006, Puerto Rico).*

Conclusions

We have found that photodynamic tumor vascular targeting induced significant vascular functional changes. As a result, tumor accumulation of fluorescence macromolecular probes (FITC-dextran, TRITC-albumin) is significantly enhanced after photodynamic vascular targeting, as demonstrated by intravital microscope and stereofluorescence microscope imaging. Immunofluorescence staining of endothelial cytoskeleton structure further indicates microtubule depolymerization, stress actin fiber formation and intercellular gap formation. The combination of photodynamic tumor vascular targeting and anticancer agents leads to a synergistic therapeutic effect. PDT is under clinical trial for the prostate cancer treatment. Our results suggest the combination of PDT with anticancer drug therapy. Our future work will focus on understanding the mechanism of such combination at molecular and tissue levels.

Appendices

Chen B, Pogue BW, Luna J, Hardman R, Hoopes PJ, Hasan T. Tumor vascular permeabilization by vascular-targeting photosensitization: effects, mechanism and therapeutic implications. *Clin Cancer Res.* 2006, 10: 917-23.

Chen B, Pogue BW, Hoopes PJ, Hasan T. Vascular and cellular targeting for photodynamic therapy. *Crit Rev Eukaryot Gene Expr.* 2006, 16: 279-306.

Tumor Vascular Permeabilization by Vascular-Targeting Photosensitization: Effects, Mechanism, and Therapeutic Implications

Bin Chen,^{1,2} Brian W. Pogue,^{2,3} Jorge M. Luna,² Rulon L. Hardman,¹
P. Jack Hoopes,^{1,2} and Tayyaba Hasan³

Abstract Purpose: Loss of vascular barrier function has been observed shortly following vascular-targeting photodynamic therapy. However, the mechanism involved in this event is still not clear, and the therapeutic implications associated with this pathophysiologic change have not been fully explored.

Experimental Design: The effect of vascular-targeting photodynamic therapy on vascular barrier function was examined in both s.c. and orthotopic MatLyLu rat prostate tumor models and endothelial cells *in vitro*, using photosensitizer verteporfin. Vascular permeability to macromolecules (Evans blue-albumin and high molecular weight dextran) was assessed with dye extraction (*ex vivo*) and intravital microscopy (*in vivo*) methods. Intravital microscopy was also used to monitor tumor vascular functional changes after vascular-targeting photodynamic therapy. The effects of photosensitization on monolayer endothelial cell morphology and cytoskeleton structures were studied with immunofluorescence staining.

Results: Vascular-targeting photodynamic therapy induced vascular barrier dysfunction in the MatLyLu tumors. Thus, tumor uptake of macromolecules was significantly increased following photodynamic therapy treatments. In addition to vascular permeability increase, blood cell adherence to vessel wall was observed shortly after treatment, further suggesting the loss of endothelial integrity. Blood cell adhesion led to the formation of thrombi that can occlude blood vessels, causing vascular shutdown. However, viable tumor cells were often detected at tumor periphery after vascular-targeting photodynamic therapy. Endothelial cell barrier dysfunction following photodynamic therapy treatment was also observed *in vitro* by culturing monolayer endothelial cells on Transwell inserts. Immunofluorescence study revealed microtubule depolymerization shortly after photosensitization treatment and stress actin fiber formation thereafter. Consequently, endothelial cells were found to retract, and this endothelial morphologic change led to the formation of intercellular gaps.

Conclusions: Vascular-targeting photodynamic therapy permeabilizes blood vessels through the formation of endothelial intercellular gaps, which are likely induced via endothelial cell microtubule depolymerization following vascular photosensitization. Loss of endothelial barrier function can ultimately lead to tumor vascular shutdown and has significant implications in drug transport and tumor cell metastasis.

Authors' Affiliations: ¹Department of Surgery, Dartmouth Medical School, Lebanon; ²Thayer School of Engineering, Dartmouth College, Hanover, New Hampshire; and ³Department of Dermatology, Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts. Received 8/1/05; revised 10/19/05; accepted 11/23/05.

Grant support: National Cancer Institute grant P01CA84203 and Department of Defense grant W81XWH-04-1-0077.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: B. Chen is currently at the Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, University of the Sciences in Philadelphia, 600 South 43rd Street, Philadelphia, PA 19104.

Requests for reprints: Brian W. Pogue, Thayer School of Engineering, 8000, Cummings Hall, Dartmouth College, Hanover, NH 03755. Phone: 215-596-7481; Fax: 215-895-1161; E-mail: pogue@dartmouth.edu.

© 2006 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-05-1673

Photodynamic therapy is a modality in which a photosensitizer is administered systemically or locally and subsequently activated by illumination with visible light, leading to the generation of cytotoxic reactive oxygen species in the presence of oxygen (1). Photodynamic therapy is currently used for the treatment of various types of cancer, including lung, skin, gastrointestinal tract, the head and neck, and urological cancers (2). It has also been used as a treatment for noncancer diseases such as age-related muscular degeneration (AMD), atherosclerosis, and viral or bacterial infections (3).

Verteporfin (the lipid formulation of benzoporphyrin derivative monoacid ring) is a photosensitizer that has been approved for the treatment of AMD (4). Compared with Photofrin (the first photosensitizer with the Food and Drug Administration approval for cancer treatment), the advantages of verteporfin include a strong absorption at longer wavelengths, leading

to deeper tissue penetration and a fast pharmacokinetic behavior *in vivo*, resulting in a reduced skin photosensitivity. Because photosensitizing targets closely depend on the localization of photosensitizers, it is therefore important to determine the temporal and spatial changes of the photosensitizer localization. In the previous studies, we have found that the distribution of verteporfin changes dynamically as a function of time after administration. It is predominantly retained in the tumor vasculature within the first few minutes after i.v. injection (e.g., within 15 minutes) and then systematically extravasates into the tumor interstitial and cellular compartments over longer times (e.g., over a few hours) after administration (5–7). Based on this pharmacokinetic pattern, maximal tumor vascular or cellular targeting can be effectively achieved by illumination at a short or a long time point after drug administration, respectively. Light treatment typically starts at 5 to 15 minutes after administration of verteporfin to selectively target blood vessels. This vascular targeting regimen is currently used for AMD treatment in clinic (4) and experimentally for tumor destruction (5, 6, 8, 9).

Vascular-targeting therapy is a promising strategy in cancer treatment that has received considerable attention in recent years (10, 11). Compared with conventional cancer cell-targeting approaches, targeting tumor vasculature is easier to access, more efficient in cancer cell killing, and has a lower likelihood of developing drug resistance. Although vascular damage has long been known to contribute to the overall photodynamic therapy treatment effect, intentional use of this mechanism based on the photosensitizer pharmacokinetic distribution to maximize clinical effect is a more recent technique, beginning with the implementation of verteporfin for AMD treatment. Following the success of verteporfin, the photodynamic therapy vascular targeting regimen with a short drug-light interval has been used for another photosensitizing agent Tookad, which is currently in clinical trials for prostate cancer treatment (12).

Tumor vasculature is not only a pipeline for the supply of nutrients and removal of metabolic wastes but also a common route for the delivery of anticancer agents to tumor tissues and dissemination of tumor cells to distant organs. The circulatory function of vasculature is largely maintained by the endothelial barrier that tightly controls the substance exchange between blood plasma and interstitial fluids (13). The goal of vascular targeting is to induce vascular shutdown. One of the earliest events following vascular photosensitization is, however, the loss of vascular barrier function (14, 15). Indeed, increase in vascular permeability has been documented after photodynamic therapy treatment with several photosensitizers (14). In AMD patients treated with verteporfin-photodynamic therapy, vascular leakage is observed shortly after treatment and lasts even for days before vessel occlusion (16). Given the critical role of vasculature in tumor cell survival, metastasis, and anticancer drug delivery, it is important to study the effects and mechanisms of verteporfin photosensitization on vascular barrier function. A fundamental understanding of photosensitization-induced vascular permeabilization is necessary for using this modality to target blood vessels for the treatment of cancer, AMD, and other diseases. In this article, we studied tumor vascular barrier function alteration and its mechanisms in response to photodynamic therapy with verteporfin, as used in a vascular-targeting approach.

Materials and Methods

Photosensitizer. Verteporfin (benzoporphyrin derivative in a lipid formulation) was obtained from QLT, Inc., as a gift (Vancouver, Canada). A stock saline solution of verteporfin was reconstituted according to the manufacturer's instructions and stored at 4°C in the dark.

Cell culture. Mouse endothelial cells SVEC4-10 (American Type Culture Collection, Manassas, VA) and R3327-MatLyLu rat prostate cancer cells were maintained in RPMI 1640 with glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 units/mL penicillin/streptomycin (Mediatech) at 37°C in a 5% CO₂ incubator.

Animals and tumor model. Male Copenhagen rats (6–8 weeks old) obtained from Charles River Laboratories (Wilmington, MA) were used throughout the study. The R3327-MatLyLu Dunning prostate tumor is an androgen-independent carcinoma, syngeneic to the Copenhagen rats, and highly metastatic to both lymph nodes and lungs (MatLyLu; ref. 17). This Dunning tumor was shown to be similar to human prostate cancer in the response to hormone therapy, chemotherapy, and radiation therapy (18). Cells used in this experiment were no more than 10 passages from the original stock in liquid nitrogen. The s.c. and orthotopic MatLyLu rat prostate cancer models were reproduced as previously described (9). Tumors were used for experiments when reaching a size of 6 to 10 mm in diameter. All animal procedures were done according to a protocol approved by the Dartmouth College Animal Care and Use Committee.

Photodynamic therapy treatments. A diode laser system (Applied Optonics, South Plainfield, NJ) with 690-nm wavelength was used throughout this study for the irradiation of *in vitro* cultured cells and MatLyLu tumors. The light was delivered through an optical fiber (140- μ m core diameter). For the *in vitro* study, SVEC4-10 cells were incubated with 200 ng/mL verteporfin for 15 minutes. After removing the drug-containing medium, cells were washed with PBS and exposed to 5 mW/cm² intensity of light for 100 or 200 seconds. Light intensity was measured by an optical power meter (Thorlabs, Inc., North Newton, NJ). For photodynamic therapy treatment of MatLyLu tumors, animals were anesthetized with an injection (i.p.) of ketamine (90 mg/kg) and xylazine (9 mg/kg) and placed on a heated blanket throughout the light treatment. The MatLyLu tumors were treated with external light illumination for 1,000 seconds at an incident fluence rate of 50 mW/cm². Verteporfin was injected i.v. at a dose of 0.25 mg/kg at 15 minutes or 1.0 mg/kg at 3 hours before light irradiation.

Assessment of vascular permeability to macromolecules in the MatLyLu tumors. Effective vascular permeability in the s.c. MatLyLu tumors was determined as described (19). Immediately after photodynamic therapy treatments (both 15 minutes and 3 hours of drug-light photodynamic therapy), animals were i.v. injected with 10 mg/kg Evans blue (Sigma, St. Louis, MO) and 10 mg/kg FITC-labeled dextran (molecule weight of 2,000 kDa; Sigma). At 0.25, 0.5, 1.0, and 2.0 hours after injection, tumor-bearing animals were euthanized. After systemic perfusion with 50 mL of 0.9% saline to remove macromolecules in the circulation, tumor tissues were excised, minced, and extracted with formamide (1 mL per 100 mg tissue) for 72 hours. The absorbance of Evans blue at 620 nm was measured with a spectrophotometer (Cary 50 Bio, Varian Analytical Instruments, Walnut Creek, CA), and the fluorescence of FITC-dextran was determined with a spectrofluorometer (FluoroMax-3, Jobin Yvon, Inc., Edison, NJ) with 495-nm excitation and 518-nm emission.

Monitoring of tumor vascular function by intravital microscopy. Tumor vascular functional changes induced by vascular-targeting photodynamic therapy regimen (light treatment at 15 minutes after injection of 0.25 mg/kg verteporfin) were examined using a Zeiss fluorescence stereomicroscope (Stemi SV11) in the live animals with orthotopic MatLyLu tumors. Tumor-bearing animals were anesthetized as described above and fixed on the stereomicroscope stage. Orthotopic MatLyLu tumors were surgically exposed and treated with 50 J/cm² dose

of light at 15 minutes after i.v. injection of 0.25 mg/kg verteporfin. Immediately after photodynamic therapy treatment, animals were injected with a 2,000-kDa FITC-dextran (10 mg/kg, i.v.). The extravasation of the 2,000-kDa FITC-dextran was imaged using a $\times 1$ objective lens with $\times 6.6$ zoom, and the fluorescence images were captured with an AxioCam CCD camera (Zeiss, Gottingen, Germany) with the filter set of 470 to 490 nm for excitation and 520 to 560 nm for emission. The camera settings were kept constant for the control and photodynamic therapy-treated animals.

To assess the effects of vascular-targeting photodynamic therapy on blood perfusion, rat red blood cells (RBC) were labeled with a fluorescence dye Dil (a carbocyanine dye) as described (20). Briefly, heparinized whole blood was collected from a donor rat. RBCs were isolated from the whole blood by centrifugation and washing with PBS thrice. Then, 1 mL of packed RBCs was incubated with 1 mL of Dil solution (1 mg/mL) at room temperature in dark condition for 30 minutes. After the incubation, RBC suspension was centrifuged and washed with PBS twice to remove the free dye. Then, 200 μ L of Dil-labeled RBCs diluted with 800 μ L PBS was i.v. injected to the animals before photodynamic therapy treatment. The movement of Dil-labeled RBCs was monitored with the stereomicroscope using a $\times 1$ objective lens plus 6.6 zoom, and the fluorescence images were recorded with the AxioCam CCD camera. The filter set for imaging Dil dye was 530 to 550 nm for excitation and 570 to 610 nm for emission.

Assessment of monolayer endothelial permeability. *In vitro* endothelial permeability was measured by the diffusion of 2,000-kDa FITC-dextran through the endothelial monolayer, as described (21). SVEC-4 endothelial cells were cultured on Transwell inserts (Costar, Cambridge, MA) up to confluence. Cells were incubated with 200 ng/mL verteporfin for 15 minutes and subjected to light treatment (100 or 200 seconds of illumination at 5 mW/cm²). Immediately after light irradiation, medium containing 1 mg/mL of 2,000-kDa FITC-dextran was loaded on the upper compartment of the Transwell. The amount of FITC-dextran diffused through the endothelial monolayer into the lower compartment was measured by a SynergyHT microplate reader (Bio-Tek Instruments, Winooski, VT) with excitation at 485/20 nm and emission at 525/20 nm.

Immunofluorescence staining of endothelial cytoskeleton. SVEC-4 endothelial cells cultured on glass coverslips were treated with 5 mW/cm² light for 200 seconds after incubation with 200 ng/mL verteporfin for 15 minutes. At different time points after treatment, cells were fixed and permeated with cold methanol/acetone (1:1) at -20°C for 30 minutes. Cells were subsequently washed thrice with PBS and blocked for nonspecific binding with 1% bovine serum albumin in PBS for 30 minutes at room temperature. The microtubule was stained with anti- α -tubulin mouse monoclonal antibody (Sigma; 1:500 dilution) for 1 hour at room temperature followed by incubation with Alexa 488-conjugated rabbit anti-mouse secondary antibody (Molecular Probes, Eugene, OR; 1:500 dilution) for 30 minutes. Actin filaments were stained with rhodamine-conjugated phalloidin (Sigma; 1 μ g/mL) for 1 hour at room temperature. Cell nuclei were stained with Hoechst dye (Sigma, 5 μ mol/L) for 15 minutes. After immunofluorescence staining, cells were imaged with a Zeiss LSM 510 confocal microscopy with appropriate filter setup for different dyes.

Results

Tumor vascular permeability to Evans blue and FITC-dextran (molecular weight, 2,000 kDa) was first assessed in the s.c. MatLyLu tumors. Figure 1 indicates that vascular targeting verteporfin-photodynamic therapy using a 15-minute drug-light interval increases vascular permeability; thus, tumor uptake of macromolecules is significantly increased at 2 hours after injection compared with the control tumor. In contrast, verteporfin-photodynamic therapy using a 3-hour drug-light interval does not significantly increase tumor uptake of the

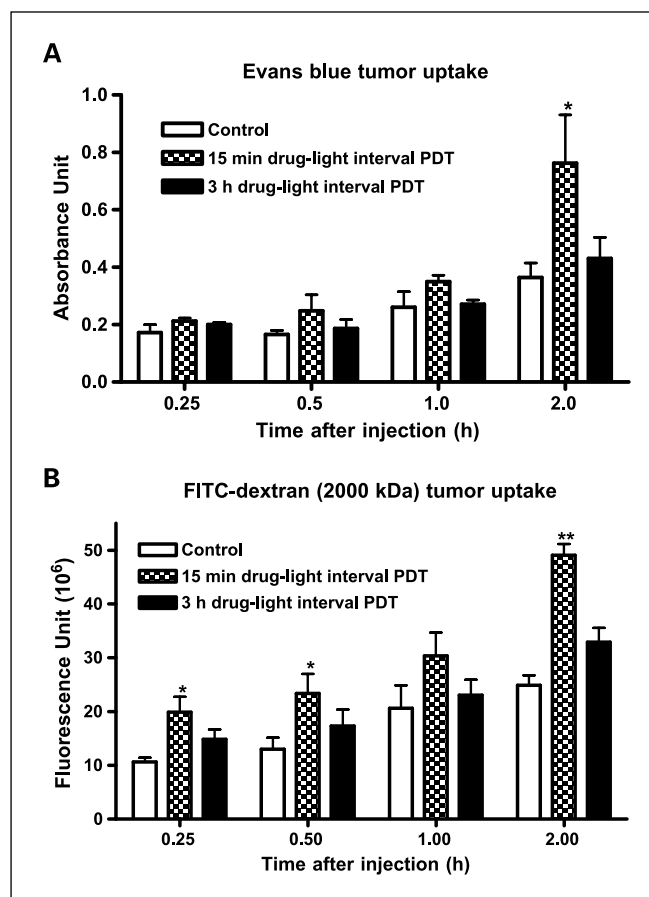


Fig. 1. Verteporfin-photodynamic therapy (PDT) increases vascular permeability to macromolecules in the subcutaneous MatLyLu rat prostate tumor model. Tumors were exposed to 50 J/cm² dose of light treatment (50 mW/cm²) at either 15 minutes following 0.25 mg/kg verteporfin injection or 3 hours after 1 mg/kg verteporfin injection. Evans blue (10 mg/kg, A) and 2,000-kDa FITC-dextran (10 mg/kg, B) were i.v. injected immediately after photodynamic therapy. Tumor uptake of Evans blue and FITC-dextran was measured with spectrophotometry and spectrofluorometry, respectively, at different time points after injection ($n = 3$). *, $P < 0.05$; **, $P < 0.01$, compared with control.

macromolecules. It was noted that vascular-targeting photodynamic therapy could significantly enhance tumor uptake of 2,000-kDa FITC-dextran at 0.25 and 0.5 hour after photodynamic therapy. However, the same treatment was not able to increase Evans blue tumor uptake (Fig. 1).

Vascular permeabilization induced by vascular-targeting photodynamic therapy could also be observed in the orthotopic tumor in real time with intravital microscopy. Immediately after photodynamic therapy treatment, animals were i.v. injected with 2,000-kDa FITC-dextran, and the extravasation of high-molecule dextran was monitored in live animals with a stereo fluorescence microscope. Because blood significantly quenches the fluorescence of FITC through the inner filter effects of hemoglobin (22), only a weak fluorescence signal could be observed within tumor blood vessels (Fig. 2). However, when FITC-dextran leaked out of blood vessels, its fluorescence intensity was greatly enhanced due to the loss of hemoglobin-quenching effect. As shown in Fig. 2, vascular-targeting photodynamic therapy permeabilizes tumor blood vessels, significantly increasing the extravasation of high molecule weight dextran, whereas the leakage of 2,000 kDa

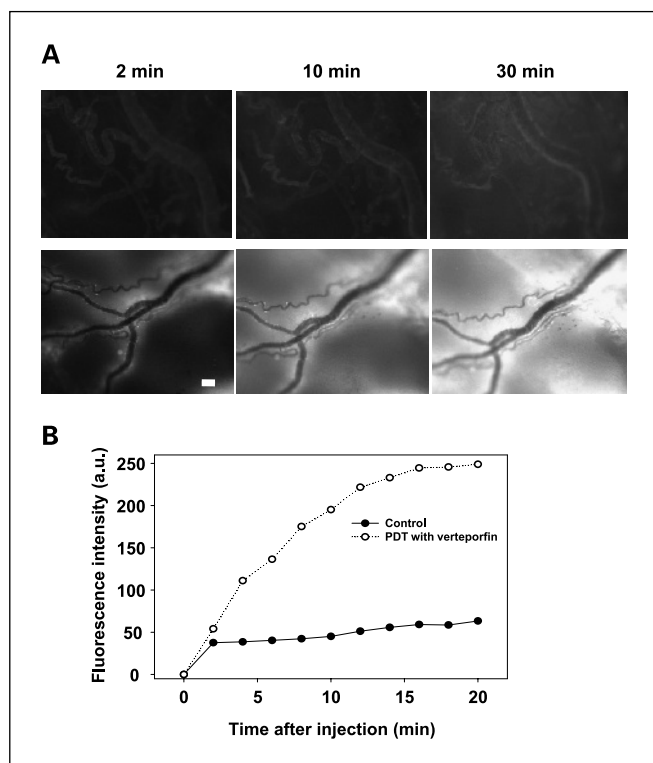


Fig. 2. *A*, intravital microscopic imaging showing the extravasation of 2,000-kDa FITC-dextran out of tumor blood vessels. Orthotopic MatLyLu rat prostate tumors were treated with vascular-targeting photodynamic therapy (PDT; i.e., 50 J/cm² dose of light; 50 mW/cm²) at 15 minutes following 0.25 mg/kg verteporfin injection. Control tumors were only injected with 0.25 mg/kg verteporfin without light treatment. Immediately after treatment, animals were injected with 2,000-kDa FITC-dextran (i.v. 10 mg/kg), and tumor blood vessels were imaged with a stereo fluorescence microscope at 2, 10, and 30 minutes thereafter. Top, control tumor; bottom, photodynamic therapy-treated tumor. Bar, 50 μ m. *B*, quantitative analysis of 2,000-kDa FITC-dextran extravasation. Total fluorescence intensity was measured with NIH ImageJ software.

in control tumors is limited. Intravital microscope study also revealed the adhesion of fluorescence-labeled RBCs to the vessel wall shortly after vascular-targeting photodynamic therapy (Fig. 3). Blood cell adherence gradually built up and led to the formation of thrombus. Some thrombi were unstable and went into circulation, leaving blood vessels still functional, whereas other thrombi remained at where they were formed and finally occluded the blood vessels. As shown in Fig. 3, an injection of 2,000-kDa FITC-dextran highlights an apparently functional blood vessel at 120 minutes after photodynamic therapy, whereas a nearby vessel occluded by a thrombus showed no fluorescence at all. It is interesting to note that the FITC fluorescence intensity in the blood vessel in this case is much stronger than that in Fig. 2, although the drug dose injected is the same. This might suggest that although still functional at 120 minutes after photodynamic therapy, that blood vessel has low hemoglobin content. A possible explanation for this observation is that photodynamic therapy-induced thrombosis and direct photodynamic damage of RBCs causes some tumor blood vessels flowed with a lower percentage of RBC volume.

Histologic examination of H&E staining tumor sections taken from tumors at 48 hours after vascular-targeting photodynamic therapy indicated extensive vascular disruption and tumor cell

death throughout tumor sections (Fig. 4). However, viable tumor cells were commonly detected at tumor periphery. Because of the existence of viable peripheral tumor cells, the vascular-targeting regimen used in this study led to no tumor cure (6).

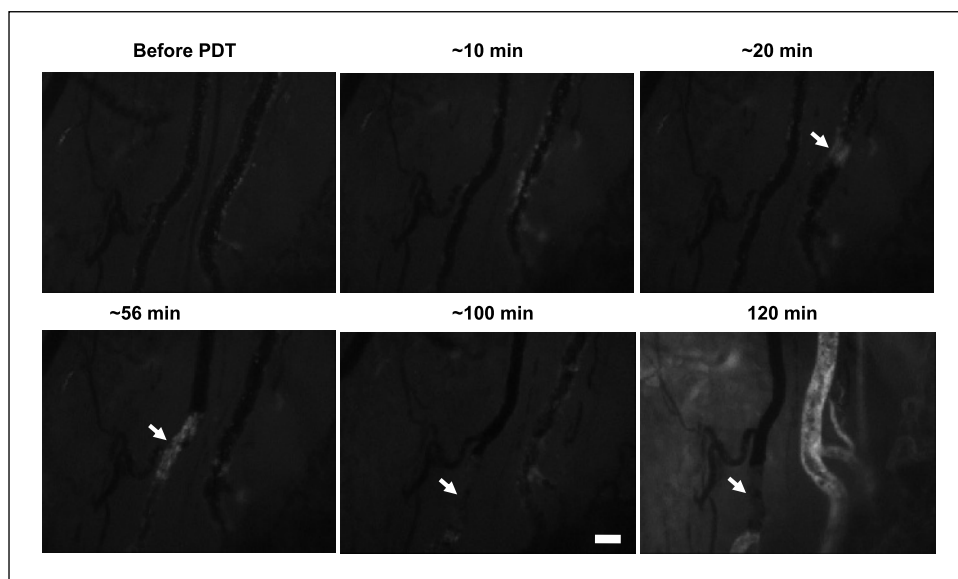
Endothelial barrier function was also assessed by the diffusion of 2,000-kDa FITC-dextran through the endothelial monolayer cultured on transwell inserts. As shown in Fig. 5, photosensitization with verteporfin (200 ng/mL for 15-minute incubation) significantly increased monolayer endothelial permeability to the macromolecule 2,000-kDa FITC-dextran in a dose-dependent manner, whereas the permeability in the control endothelial cells (with 200 ng/mL verteporfin only, no light) was very limited.

Changes in endothelial cytoskeleton induced by verteporfin-photodynamic therapy were examined with immunofluorescence staining. In the control cells, microtubules extend throughout the cytoplasm to the cell periphery, whereas actin only distributes at the cell periphery (Fig. 6, top). This distribution pattern is important for maintaining endothelium integrity (23). Microtubule disassembly was noted shortly after verteporfin-photodynamic therapy followed by the formation of actin stress fibers located in the cell central region (Fig. 6, middle). Accompanying the actin stress fibers formation, endothelial cells were observed to retract and display a round morphology, leading to the formation of intercellular gaps (Fig. 6, bottom).

Discussion

The goal of tumor vascular targeting is to selectively modulate tumor vascular function for a therapeutic purpose (24). To achieve this goal, therapeutic effectors or cytotoxic agents need to be selectively delivered to the tumor vascular targets. Although there are a variety of potential tumor vascular markers that can be exploited for the selective vascular targeting through conjugating therapeutic agents with tumor vasculature homing molecules, a marker that is absolutely specific for tumor vasculature has not yet been and may never be found (25). Passive targeting of tumor vasculature based upon the temporal confinement of an i.v. injected agent might be practically the most effective approach to targeting tumor blood vessels. This is especially true for photodynamic therapy, where light needs to be applied to activate the photosensitizing compounds that are otherwise not biologically active at all. Photodynamic therapy can be developed as an effective and selective vascular-targeting modality because photosensitizers are exclusively localized within the vasculature shortly after systemic administration and, more importantly, the selectivity of action to the desired site comes through the ability to accurately deliver light provided by current laser fiber technology. Indeed, photodynamic vascular-targeting therapy has already been in clinical applications for AMD and is under clinical investigation for cancer treatments. However, in spite of extensive studies, a detailed scenario of how photodynamic therapy causes vascular shutdown remains unclear. The present study focuses on studying the effects and mechanisms of vascular permeabilization, an early event commonly observed after photodynamic vascular-targeting therapy.

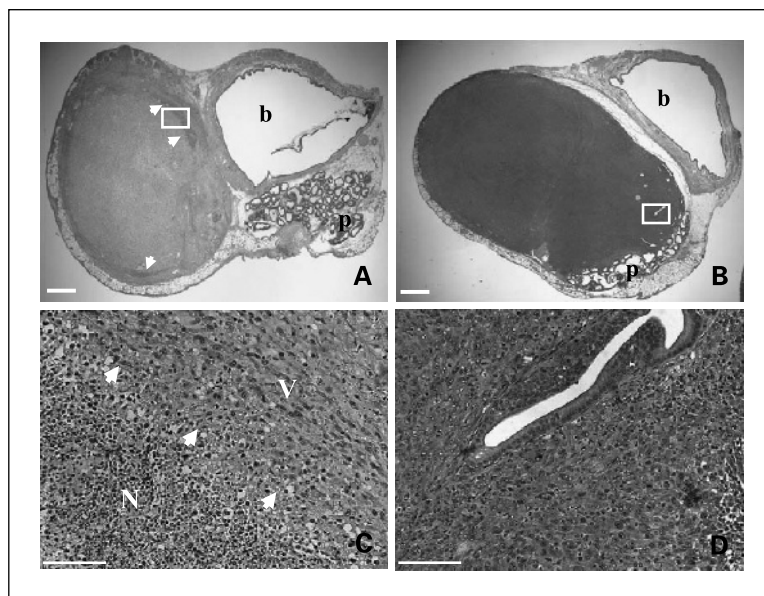
Fig. 3. Intravital microscopic imaging of tumor vascular response to vascular-targeting photodynamic therapy in the orthotopic MatLyLu rat prostate tumor. Rat blood cells were labeled with Dil dye as described in the Materials and Methods and injected to the animals. The orthotopic MatLyLu tumors were exposed to 50 J/cm² light (690 nm, at 50 mW/cm²) at 15 minutes after i.v. injection of 0.25 mg/kg verteporfin. Fluorescence images of tumor blood vessels indicate blood cell adherence and thrombus formation (*arrow*). To examine the vascular function at 120 minutes after photodynamic therapy, the animal was i.v. injected with 10 mg/kg 2,000-kDa FITC-dextran. Fluorescence of FITC was observed in the remaining functional vessels. Bar, 50 μ m.



Our present results show that photosensitization with verteporfin significantly increases overall vascular permeability in both s.c. and orthotopic MatLyLu rat prostate tumors. Thus, tumor uptake of macromolecules was increased after the initial photosensitization treatment. This effect seems dependent on the photodynamic therapy conditions and the size of macromolecules. A vascular-targeting photodynamic therapy regimen employing a short drug-light interval induced a stronger effect than the cellular-targeting photodynamic therapy using a long interval (Fig. 1). This is likely because vascular barrier function is maintained by the integrity of endothelial network and specific intravascular photosensitization induced by vascular-targeting photodynamic therapy is able to induce more structural and functional changes on the endothelium. Previous studies also showed that vascular targeting photodynamic therapy employing a short drug-light interval caused more

reduction in blood flow (5, 8, 26). It is interesting to note that the increase in tumor uptake of 2,000 kDa dextran was more significant than that of Evans blue. This difference might be related to the size of these two macromolecules. Evans blue strongly binds to albumin in the blood. Its behavior reflects the transport of albumin (19), which is about 67 kDa with a diameter of about 7 nm. This size is similar to the effective pore size of 6 to 7 nm occurring in most normal blood vessels (27), whereas the size of 2,000-kDa dextran is estimated to be about 100 nm (28). Because tumor vessels typically have larger interendothelial junctions than normal blood vessels (29), there might be little hindrance for the transvascular transport of Evans blue-albumin complex. Therefore, further increase in vascular permeability induced by vascular photosensitization may have little influence on the extravasation of albumin that can already cross tumor vessel wall. However, it can

Fig. 4. Histologic changes of orthotopic MatLyLu tumor after photodynamic therapy treatment. The MatLyLu tumors were exposed to 50 J/cm² light treatment (690 nm, at 50 mW/cm²) at 15 minutes after i.v. injection of 0.25 mg/kg verteporfin. Control tumors were only injected with verteporfin without light treatment. Tumor sections were taken at 48 hours after treatments and stained with H&E. Photographs (A) and (C) were taken from a photodynamic therapy-treated tumor section, and photographs (B) and (D) were from a control tumor section. Photographs (A) and (B) were taken at a low magnification, showing a complete tumor section, including the tumor, prostate (p), and bladder (b). Part of the tumor section (*white box*) in photographs (A) and (B) is highlighted at a high magnification in photographs (C) and (D), respectively. Note a clear demarcation (*arrow*) between necrotic tumor area (N) and viable tumor area (V) at tumor peripheral region. Bar, 1 mm (A and B) and 100 μ m (C and D).



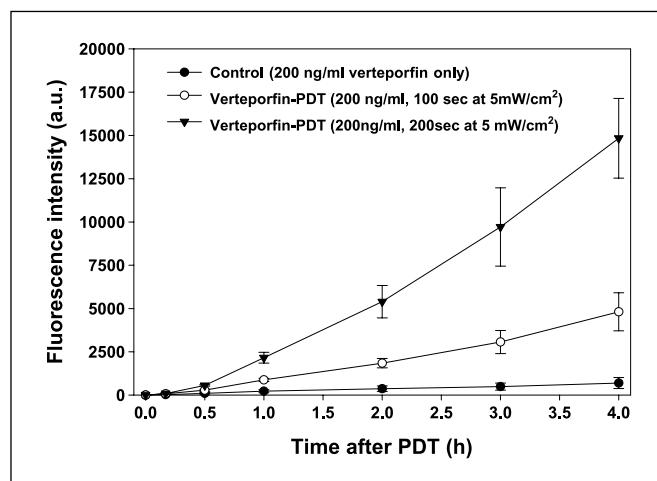


Fig. 5. Photosensitization with verteporfin induces an increase in endothelial monolayer permeability to 2,000-kDa FITC-dextran. Confluent SVEC4-10 endothelial cells cultured on Transwell inserts were exposed to 5 mW/cm² light treatment for 100 or 200 seconds after incubation with 200 ng/mL verteporfin in medium for 15 minutes. The amount of FITC-dextran diffused through the endothelial monolayer into the lower compartment was measured by a microplate reader with excitation at 485/20 nm and emission at 525/20 nm. Control cells were only incubated with 200 ng/mL verteporfin without light treatment. PDT, photodynamic therapy.

significantly facilitate the extravasation of larger molecules, such as 2,000-kDa dextran, that are otherwise difficult to transport across the endothelial barrier.

The mechanism of photosensitization-induced vascular permeabilization is still an unresolved issue. Because vascular barrier function critically depends on the endothelial cell integrity, which is maintained by cytoskeletal components, such as filament actin and microtubules (13), we studied the effects of verteporfin photosensitization on endothelial cell morphology, cytoskeleton, and barrier function. Our results show that photosensitization causes endothelial cell microtubule depolymerization and induces the formation of actin stress fibers (Fig. 6). Thus, endothelial cells were found to retract, leading to the formation of intercellular gaps, which result in endothelial barrier dysfunction (Fig. 5). The key question becomes how photosensitization induces the formation of intercellular gaps. Here, we found that microtubule alteration was noted before any apparent changes of actin structures and cell morphology, suggesting that microtubules play a pivotal role in photosensitization-induced endothelial gap formation. Microtubules are a cytoskeletal structure with important function in signal transduction and intracellular transport of membrane-bound organelles (23). Previous study with Photofrin also showed that microtubules were even sensitive to photodynamic therapy dose that produced little cytotoxicity (30). It is likely that direct photodynamic disruption of microtubule network triggers endothelium contraction by inducing actin cytoskeletal changes, such as the formation of actin stress fiber, a filament with contractile property. It has been shown that microtubule disruption can cause endothelial morphologic changes through the activation of Rho protein (31). We are currently investigating the involvement of Rho/Rho kinase pathway in photosensitization-induced endothelial morphologic and functional changes.

Retraction of endothelial cells not only leads to the formation of intercellular gap and therefore causes vascular barrier dysfunction but also exposes basement membrane to circulating blood cells, which triggers blood aggregation cascade and causes blood flow reduction. Our intravital microscopy study showed RBC adherence to vessel wall shortly after vascular-targeting photodynamic therapy (Fig. 3). Blood cell adherence developed into the formation of thrombi. Stable thrombi would decrease blood flow and eventually occlude blood vessels, as shown in Fig. 3. This is in agreement with electron microscopic study showing that tumor blood vessels are often congested with RBCs after photodynamic therapy treatment (32). Exposure of vessel basement membrane as a result of endothelial retraction might be only one of the mechanisms causing thrombi formation. Other mechanisms, such as release of thromboxane from platelets (33) and von Willebrand factor from damaged endothelial cells (34), could also contribute to the thrombosis process.

Because tumor vascular leakiness, on the one hand, governs the delivery of therapeutic agents into the tumor tissue and, on the other hand, facilitates tumor cell intravasation into the circulation (35), tumor vascular permeabilization induced by vascular-targeting photodynamic therapy has profound implications in cancer treatments. A therapeutic benefit of photosensitized vascular permeabilization is that it can be used to improve tumor drug delivery and enhance the therapeutic

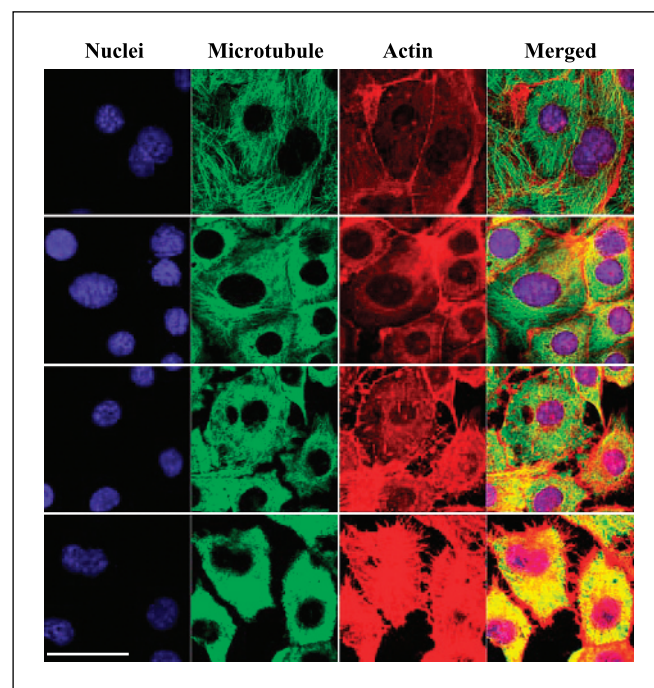


Fig. 6. Photodynamic therapy with verteporfin induces microtubule depolymerization and stress actin fiber formation in SVEC4-10 endothelial cells (with objective lens of $\times 40$). Cells cultured on glass coverslips were treated with 5 mW/cm² light for 200 seconds after incubation with 200 ng/mL verteporfin for 15 minutes. Nuclei were stained with Hoechst (blue). Microtubule was stained with anti-tubulin antibody followed by incubation with Alexa Fluor 488-labeled secondary antibody (green), and filament actin was stained with rhodamine-phalloidin (red). Merged images of all three staining (right). Top, control; middle top, 5 minutes after photodynamic therapy; middle bottom, 15 minutes after photodynamic therapy; bottom, 30 minutes after photodynamic therapy. Bar, 10 μ m.

effect. Indeed, it has been shown that photodynamic therapy regimens with low fluence and fluence rate are able to induce a significant increase in tumor vascular permeability for a sustained period of time (36). Consequently, combination of these photodynamic therapy treatments with liposomal doxorubicin led to an enhanced tumor cure. On the other hand, because tumor vasculature represents an interface between the circulation system and cancer cells, a concern of photodynamic therapy-induced vascular permeabilization is that whether this can potentially induce tumor metastasis by increasing tumor cell intravasation into the circulation. There is evidence showing that sublethal photodynamic therapy damage to tumor cells indeed increases tumor metastasis (37). Although this is considered to be related to the decrease of

tumor cell adhesion to the extracellular matrix and the activation of tumor cell survival signal (such as expression of hypoxia-inducible factor-1 α and vascular endothelial growth factor) following sublethal photodynamic therapy damage to tumor cells, tumor vascular permeability increase may at least contribute to the metastatic process because sublethal photodynamic therapy itself together with some tumor secreting factors (e.g., vascular endothelial growth factor) all can increase tumor vascular leakiness. Thus, our future efforts will be on exploring the mechanism and therapeutic potential of photodynamic vascular targeting in cancer therapy and anticancer drug delivery, and, importantly, addressing the concern of whether photosensitized vascular permeabilization will increase tumor metastasis.

References

1. Dougherty TJ, Gomer CJ, Henderson BW, et al. Photodynamic therapy. *J Natl Cancer Inst* 1998;90:889–905.
2. Dolmans DE, Fukumura D, Jain RK. Photodynamic therapy for cancer. *Nat Rev Cancer* 2003;3:380–7.
3. Detty MR, Gibson SL, Wagner SJ. Current clinical and preclinical photosensitizers for use in photodynamic therapy. *J Med Chem* 2004;47:3897–915.
4. Brown SB, Mellich KJ. Verteporfin: a milestone in ophthalmology and photodynamic therapy. *Expert Opin Pharmacother* 2001;2:351–61.
5. Chen B, Pogue BW, Goodwin IA, et al. Blood flow dynamics after photodynamic therapy with verteporfin in the RIF-1 tumor. *Radiat Res* 2003;160:452–9.
6. Chen B, Pogue BW, Hoopes PJ, Hasan T. Combining vascular and cellular targeting regimens enhances the efficacy of photodynamic therapy. *Int J Radiat Oncol Biol Phys* 2005;61:1216–26.
7. Zhou X, Pogue BW, Chen B, Hasan T. Analysis of effective molecular diffusion rates for verteporfin in subcutaneous versus orthotopic Dunning prostate tumors. *Photochem Photobiol* 2004;79:323–31.
8. Finger VH, Kik PK, Haydon PS, et al. Analysis of acute vascular damage after photodynamic therapy using benzoporphyrin derivative (BPD). *Br J Cancer* 1999;79:1702–8.
9. Chen B, Pogue BW, Zhou X, et al. Effect of tumor host microenvironment on photodynamic therapy in a rat prostate tumor model. *Clin Cancer Res* 2005;11:720–7.
10. Tozer GM, Bicknell R. Therapeutic targeting of the tumor vasculature. *Semin Radiat Oncol* 2004;14:222–32.
11. Tozer GM, Kanthou C, Baguley BC. Disrupting tumour blood vessels. *Nat Rev Cancer* 2005;5:423–35.
12. Weersink RA, Bogaards A, Gertner M, et al. Techniques for delivery and monitoring of TOOKAD (WST09)-mediated photodynamic therapy of the prostate: clinical experience and practicalities. *J Photochem Photobiol B* 2005;79:211–22.
13. Stevens T, Garcia JG, Shasby DM, Bhattacharya J, Malik AB. Mechanisms regulating endothelial cell barrier function. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L419–22.
14. Finger VH. Vascular effects of photodynamic therapy. *J Clin Laser Med Surg* 1996;14:323–8.
15. Henderson BW, Dougherty TJ. How does photodynamic therapy work? *Photochem Photobiol* 1992;55:145–57.
16. Michels S, Schmidt-Erfurth U. Sequence of early vascular events after photodynamic therapy. *Invest Ophthalmol Vis Sci* 2003;44:2147–54.
17. Tennant TR, Kim H, Sokoloff M, Rinker-Schaeffer CW. The Dunning model. *Prostate* 2000;43:295–302.
18. Lucia MS, Bostwick DG, Bosland M, et al. Workgroup I: rodent models of prostate cancer. *Prostate* 1998;36:49–55.
19. Graff BA, Bjornas I, Rofstad EK. Microvascular permeability of human melanoma xenografts to macromolecules: relationships to tumor volumetric growth rate, tumor angiogenesis, and VEGF expression. *Microvasc Res* 2001;61:187–98.
20. Unthank JL, Lash JM, Nixon JC, Sidner RA, Bohlen HG. Evaluation of carbocyanine-labeled erythrocytes for microvascular measurements. *Microvasc Res* 1993;45:193–210.
21. Vouret-Craviari V, Boquet P, Pouyssegur J, Van Obberghen-Schilling E. Regulation of the actin cytoskeleton by thrombin in human endothelial cells: role of Rho proteins in endothelial barrier function. *Mol Biol Cell* 1998;9:2639–53.
22. Lu W, Schroit AJ. Vascularization of melanoma by mobilization and remodeling of preexisting latent vessels to patency. *Cancer Res* 2005;65:913–8.
23. Lee TY, Gotlieb AI. Microfilaments and microtubules maintain endothelial integrity. *Microsc Res Tech* 2003;60:115–27.
24. Siemann DW, Chaplin DJ, Horsman MR. Vascular-targeting therapies for treatment of malignant disease. *Cancer* 2004;100:2491–9.
25. Brekken RA, Li C, Kumar S. Strategies for vascular targeting in tumors. *Int J Cancer* 2002;100:123–30.
26. Kurohane K, Tominaga A, Sato K, North JR, Namba Y, Oku N. Photodynamic therapy targeted to tumor-induced angiogenic vessels. *Cancer Lett* 2001;167:49–56.
27. Lum H, Malik AB. Regulation of vascular endothelial barrier function. *Am J Physiol* 1994;267:L223–41.
28. Yuan H, Gaber MW, McColgan T, Naimark MD, Kiani MF, Merchant TE. Radiation-induced permeability and leukocyte adhesion in the rat blood-brain barrier: modulation with anti-ICAM-1 antibodies. *Brain Res* 2003;969:59–69.
29. Roberts WG, Palade GE. Neovascularity induced by vascular endothelial growth factor is fenestrated. *Cancer Res* 1997;57:765–72.
30. Sporn LA, Foster TH. Photofrin and light induces microtubule depolymerization in cultured human endothelial cells. *Cancer Res* 1992;52:3443–8.
31. Birukova AA, Birukov KG, Smurova K, et al. Novel role of microtubules in thrombin-induced endothelial barrier dysfunction. *FASEB J* 2004;18:1879–90.
32. Peng Q, Nesland JM. Effects of photodynamic therapy on tumor stroma. *Ultrastruct Pathol* 2004;28:333–40.
33. Finger VH, Wieman TJ, Wiehle SA, Cerrito PB. The role of microvascular damage in photodynamic therapy: the effect of treatment on vessel constriction, permeability, and leukocyte adhesion. *Cancer Res* 1992;52:4914–21.
34. Foster TH, Primavera MC, Marder VJ, Hilf R, Sporn LA. Photosensitized release of von Willebrand factor from cultured human endothelial cells. *Cancer Res* 1991;51:3261–6.
35. McDonald DM, Baluk P. Significance of blood vessel leakiness in cancer. *Cancer Res* 2002;62:5381–5.
36. Snyder JW, Greco WR, Bellnier DA, Vaughan L, Henderson BW. Photodynamic therapy: a means to enhanced drug delivery to tumors. *Cancer Res* 2003;63:8126–31.
37. Momma T, Hamblin MR, Wu HC, Hasan T. Photodynamic therapy of orthotopic prostate cancer with benzoporphyrin derivative: local control and distant metastasis. *Cancer Res* 1998;58:5425–31.

Vascular and Cellular Targeting for Photodynamic Therapy

Bin Chen,¹ Brian W. Pogue,^{2,3} P. Jack Hoopes,^{2,4} & Tayyaba Hasan^{3,*}

¹Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, University of the Sciences in Philadelphia, Philadelphia, PA 19104; ²Thayer School of Engineering, Dartmouth College, Hanover, NH 03755; ³Wellman Center for Photomedicine, Massachusetts General Hospital, Department of Dermatology, Harvard Medical School, Boston, MA 02114; ⁴Department of Surgery, Dartmouth Medical School, Lebanon, NH 03756

* Author to whom all correspondence should be addressed: E-mail: thasan@partners.org

ABSTRACT: Photodynamic therapy (PDT) involves the combination of photosensitizers (PS) with light as a treatment, and has been an established medical practice for about 10 years. Current primary applications of PDT are age-related macular degeneration (AMD) and several types of cancer and precancer. Tumor vasculature and parenchyma cells are both potential targets of PDT damage. The preference of vascular versus cellular targeting is highly dependent upon the relative distribution of photosensitizers in each compartment, which is governed by the photosensitizer pharmacokinetic properties and can be effectively manipulated by the photosensitizer drug administration and light illumination interval (drug-light interval) during PDT treatment, or by the modification of photosensitizer molecular structure. PDT using shorter PS-light intervals mainly targets tumor vasculature by confining photosensitizer localization within blood vessels, whereas if the sensitizer has a reasonably long pharmacokinetic lifetime, then PDT at longer PS-light intervals can induce more tumor cellular damage, because the photosensitizer has then distributed into the tumor cellular compartment. This passive targeting mechanism is regulated by the innate photosensitizer physicochemical properties. In addition to the passive targeting approach, active targeting of various tumor endothelial and cellular markers has been studied extensively. The tumor cellular markers that have been explored for active photodynamic targeting are mainly tumor surface markers, including growth factor receptors, low-density lipoprotein (LDL) receptors, transferrin receptors, folic acid receptors, glucose transporters, integrin receptors, and insulin receptors. In addition to tumor surface proteins, nuclear receptors are targeted, as well. A limited number of studies have been performed to actively target tumor endothelial markers (ED-B domain of fibronectin, VEGF receptor-2, and neuropilin-1). Intracellular targeting is a challenge due to the difficulty in achieving sufficient penetration into the target cell, but significant progress has been made in this area. In this review, we summarize current studies of vascular and cellular targeting of PDT after more than 30 years of intensive efforts.

KEY WORDS: photodynamic therapy (PDT), photosensitizer, vascular targeting, cellular targeting, targeted therapy, drug delivery

I. INTRODUCTION

Photodynamic therapy (PDT) is a treatment modality using light-sensitive drugs (photosensitizers) in combination with nonthermal light activation to achieve selective tissue/cell damage. PDT was initially developed for killing cancer cells. After more than a 30-year effort, Photofrin, a partially purified preparation of haemato-porphyrin derivative, became the first photosen-

sitizer approved in the United States, the European Union (EU), and many other countries for the palliative treatment of cancer and precancer diseases. Probably the most successful PDT application is not targeting cancer cells, but targeting the tumor vasculature. PDT with a liposomal photosensitizer verteporfin has received worldwide approval and become the standard of care for neovascularization involved in age-related macular degeneration (AMD). PDT can be de-

signed to target tumor cells or blood vessels. Its targeting specificity depends on the selective delivery of the photosensitizer and light to the target tissue. Current laser fiber technology allows easily controllable and highly accurate light delivery to almost any tissue in the body, although accurate dosimetry in routine practice still remains somewhat elusive. However, the progress in identifying new photosensitizers, tissue-specific markers, and targeted drug delivery systems (DDS) can significantly enhance the overall ability to selectively deposit a photosensitizer in the target site at appropriate therapeutic levels. In this review, the principle of PDT and current studies involving PDT as a means to specifically target cells and blood vessels is discussed. The application focus is largely on cancer and AMD treatment because these are the two major applications of PDT in current medical practice.

II. OVERVIEW OF PHOTODYNAMIC THERAPY

PDT relies on photophysical principles and uses photochemical reactions to generate biological effectors, such as reactive oxygen species (ROS), which cause oxidative damage to important biological molecules (proteins, lipids, and nucleic acids) in the cell membrane, cytoplasm, and nucleus.¹ It is doubtful that the damage is truly localized in any manner, but rather more likely that it is widespread in the cell and localized mainly by photosensitizer distribution. During this process, three key components—a photosensitizer, light, and oxygen—should be present simultaneously in adequate amounts to produce biological effects. A lack of any of these components will diminish or even completely abolish the effect of PDT on therapeutic outcome.

A. Photophysics and Photochemistry

Upon the absorption of photons of a suitable wavelength, individual monomer photosensitizer molecules are first excited to their short-lived excited singlet state (S_1) (lifetime typically < 100 ns) and then, through intersystem crossing, shift to a lower energy and longer lived excited triplet

state (T_1) (lifetime typically > 500 ns).² These molecules are chosen or designed to have sufficient magnetic moment to produce a triplet state splitting, which leaves the T_1 level near resonance with the excited state level of molecular oxygen. This situation then allows effective collisional quenching by ground state molecular oxygen (with the ground state already in its triplet state), to transfer energy to create singlet state oxygen. Since only triplet state molecules have a long enough lifetime to react with substrate molecules such as oxygen and generate biological effects, the quantum yield of the excited triplet state is an important criterion in evaluating the biological efficiency of photosensitizers. The triplet quantum yield of most current photosensitizers is high and ranges from about 0.3 to 0.6. The resultant triplet photosensitizer molecules may transfer electrons/hydrogen to nearby biomolecules (Type I reaction), generating free radicals that can further react with oxygen to produce reactive oxygen species (ROS).¹ It is largely believed that the majority of photosensitizers transfer their triplet state energy to oxygen via collisional quenching (Type II reaction), leading to the production of singlet oxygen. Although it is generally accepted that the Type II reaction is the dominant photochemical pathway in photodynamic reactions, as shown in vitro and with indirect studies, it is challenging to directly prove what the exact photochemical origin of the damage truly is.³ Recent studies have clearly shown that singlet oxygen is produced in vivo and that the production rate is correlated to the damage observed.⁴ However, different photosensitizers clearly have varying photochemical pathways, and it is likely that a large cascade of photochemical events is the origin of the resulting damage observed.

B. Photosensitizers

Photosensitizers are chemicals that are able to absorb photons and transfer light energy into the production of ROS, mainly singlet oxygen. Most of the current photosensitizers have porphyrin-related structures, including hematoporphyrin derivatives, phthalocyanines, chlorines, and bacteriochlorins.⁵ They can be exogenously administered compounds or endogenously produced

photosensitive metabolites (e.g., protoporphyrin IX from 5-aminolevulinic acid). To capture photons efficiently, photosensitizing compounds typically have several unsaturated aromatic rings forming large π -bond conjugation structures. Therefore, photosensitizers are generally hydrophobic and form aggregates easily in aqueous media, which not only makes intravenous administration difficult, but also decreases photodynamic efficiency. Generally, when the molecules are in monomer form they are most photophysically active, and when they dimerize or aggregate, their ability to undergo intersystem crossing is severely reduced and largely eliminated. When bound to proteins or lipids *in vivo*, they are thought to be as close to a monomer form as is feasible. It is challenging to measure the photophysical properties *in vivo*. However, with diffuse reflectance spectroscopy, some measurements have been performed in tissue, which indicate that the molecules do transition through the triplet state energy level *in vivo*. To overcome the problem of aggregation during administration, photosensitizers are generally either formulated in various colloidal drug delivery systems, such as liposomes, micelles, and biodegradable nanoparticles, or conjugated with hydrophilic polymers.⁶ It is likely that although some level of aggregation might exist, both at the time of administration and *in vivo*, the fraction of monomerized and singularly bound molecules mediate the effective photodynamic action *in vivo*.

C. Light

Light is needed to activate the photosensitizer molecules accumulated in the target tissue following administration. Although various lamp light sources can be used for this purpose, a laser light source is generally preferred due to its superior optical properties (collimation, coherence, and monochromaticity) and flexibility in manipulation in terms of delivery via small fiber optics.⁷ To achieve the highest photosensitizing efficiency, the laser wavelength should match the maximum absorption of the photosensitizer. However, since tissue endogenous molecules (e.g., hemoglobins) have strong absorption at wavelengths below 620 nm and above 900 nm (water), the most penetrat-

ing light for PDT is between these two wavelength bounds. Chemicals with long wavelength absorption (>800 nm) tend to have low production of singlet oxygen because the triplet state level is then lying below that of the singlet oxygen energy level, thereby inhibiting collisional quenching of the molecules by oxygen. Most porphyrin-based photosensitizers have two major absorption bands, with the dominant one being a Soret band near 350–450 nm in wavelength and 50–100 nm wide.⁸ The molecules with large distributed π -bond structures also have significant Q-band absorption in the red and near-infrared range of wavelengths. The basic porphyrins have a series of Q-bands all the way from 500 nm up to 630 nm, and molecules that have more distributed and asymmetric rings can have enhanced Q-bands in the 660–800 nm range. Chlorins, bacteriochlorins, phthalocyanines, and texaphyrins are all in this category.⁵ The 620- to 800-nm-wavelength range is often called the therapeutic window; light penetration is proportional to the incident light wavelength: the longer the wavelength, the deeper the tissue penetration. Therefore, red and far red light is generally used for treating bulk tissues. In certain circumstances, when a superficial treatment is highly desirable, such as in the skin, esophagus, or bladder, research has been undertaken to compare blue light excitation to red light excitation,⁹ and it is largely true that the depth of damage can be constrained by the use of blue light, whereas infrared light-activated molecules allow the maximum depth of treatment in PDT.

D. Oxygen

Because PDT uses ROS (mainly singlet oxygen) to induce irreversible cellular damage, oxygen is therefore absolutely necessary for an effective treatment. Numerous *in vivo* and *in vitro* studies have demonstrated that lack of oxygen will certainly diminish PDT effect, whereas oxygen enhancement or preservation during treatment will increase PDT efficacy, presumably as a result of enhancement in ROS production.^{10–13} Enhancement of oxygen is also beneficial in tumors that are chronically hypoxic, although systematic use of this method of enhanced sensitization is not in

practice. Since ROS have very short lifetimes and limited migration distance, the incited biological effects are largely confined to where they are produced, which is dependent on the localization of the photosensitizers. Thus, since oxygen is pervasive throughout almost all tissues, it is largely the localization of the photosensitizer that determines the areas of damage within the tissue.

III. VASCULAR TARGETING WITH PDT

Similar to normal tissue, tumor growth depends on a functional vascular system for the delivery of oxygen and nutrients and removal of metabolic wastes. However, unlike normal tissue, tumor tissue needs to keep on generating new blood vessels to maintain rapid tumor cell proliferation. Abnormally enhanced neovascularization is a hallmark of pathological conditions, such as cancer, AMD, arthritis, and diabetic retinopathy. Therefore, selectively targeting existing blood vessels (vascular-disrupting therapy) and/or inhibiting the formation of new blood vessels (antiangiogenic therapy) will have tremendous treatment effects.¹⁴ In cancer therapy, the recognition and clinical application of tumor vasculature as a therapeutic target represents a major step in cancer treatment history. Compared to conventional cancer cell-targeting approaches, the advantages of a vascular-targeting strategy includes easier accessibility, more efficient cancer cell-killing ability, and lower chance of developing therapy resistance.¹⁵ PDT has been known for many years to be able to induce strong vascular effects, which contribute significantly to the final treatment outcome.¹¹ Recent progress further demonstrate that PDT can be developed as a potent and selective vascular-targeting modality with diverse medical applications. To elicit specific photodynamic damage to the vasculature, the photosensitizing agent should be selectively distributed in the vascular compartment, which can be achieved via either passive or active targeting approaches.

A. Passive Vascular-Targeting PDT

Passive photodynamic vascular targeting refers to a vascular-targeting approach based on the accumulation of photosensitizers in the vascular

compartment as a result of pharmacological or physicochemical factors. For most exogenous photosensitizers, there is usually a peak plasma concentration immediately after intravenous administration, followed by a fast exponential decay in plasma drug level. The time period when the injected photosensitizer is largely confined in the blood vessels, generally at short time points after administration, provides a temporal therapeutic window for vascular targeting (Fig. 1). Light treatment during this vascular-targeting window, with a high plasma photosensitizer level, leads to potent vascular damage, including damage to blood cells, endothelial cells, and vessel-supporting structures. Photosensitizer physicochemical properties also contribute to the passive vascular-targeting effect. As most photosensitizers are hydrophobic, they need to be associated with plasma proteins to be transported. It has been shown that many photosensitizers bind to low-density lipoproteins (LDL) in the circulation.¹⁶ Neovascular endothelial cells and tumor cells generally have a high expression of LDL receptors resulting from increased cell proliferation. Thus, hydrophilic photosensitizers bound to LDL can be preferentially accumulated into proliferating endothelial cells through the LDL receptor-mediated endocytosis pathway.¹⁷

As a dominant mechanism underlying current clinical applications of verteporfin and other photosensitizers under clinical trials for AMD, the passive photodynamic vascular-targeting approach can be considered the most successful PDT application, so far. Table 1 summarizes the most studied passive vascular-targeting photosensitizers to date. Being the only photosensitizer that has received approval worldwide for AMD, verteporfin (benzoporphyrin derivative monoacid ring A, Visudyne) is formulated as a unilamellar liposome to aid solubility. It is found that this liposomal formulation also promotes drug redistribution to LDL,¹⁸ leading to cellular uptake via the LDL receptor-mediated endocytosis pathway.¹⁹ The average plasma half-life of verteporfin is 2–5 hours in mice and 5–6 hours in humans.¹⁷ However, to induce significant vascular destruction to choroidal neovascularization (CNV), without major damage to normal surrounding tissue caused by drug extravasation, light is generally delivered at 5–15 minutes after administration in both clinical and preclinical AMD treatments.

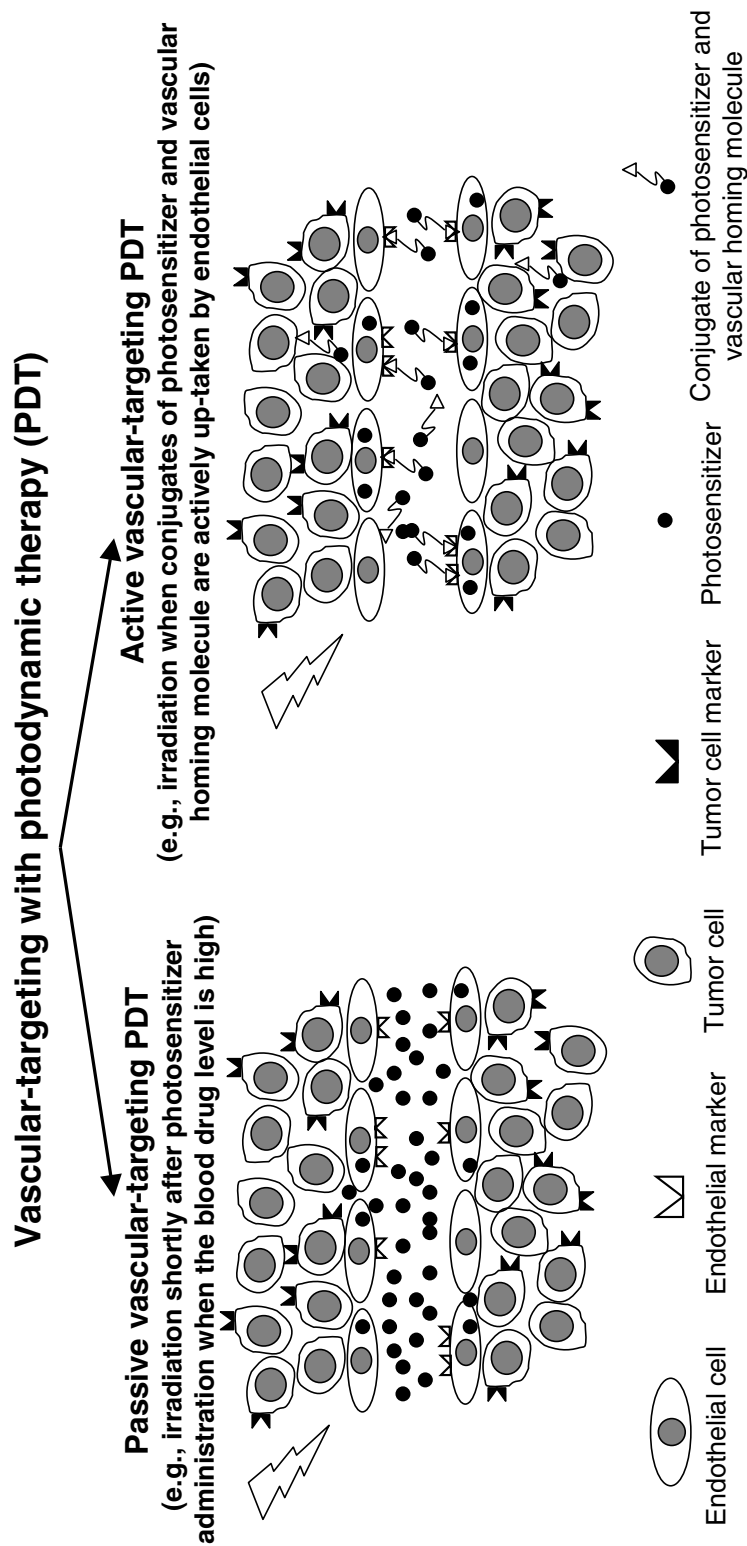


FIGURE 1. Vascular targeting with photodynamic therapy (PDT). Depending on whether a vascular homing molecule is used in the preparation of photosensitizers, vascular-targeting PDT can be divided into passive and active vascular-targeting PDT. In passive vascular-targeting PDT, preferential photosensitizer accumulation in the vascular compartment results from photosensitizer innate pharmacological or physicochemical factors, for example, high blood photosensitizer concentration shortly after photosensitizer i.v. injection. This passive vascular-targeting mechanism is primarily responsible for the clinical application of photosensitizers such as verteporfin and Tookad. In active vascular-targeting PDT, photosensitizer structural modification or targeted drug delivery system formulation with a vascular homing molecule is necessary so that photosensitizing agents can be selectively bound to and retained in the tumor vascular compartment, therefore actively targeting tumor vasculature.

TABLE 1
Common Photosensitizers Used for AMD and Cancer Treatments Based on Passive Vascular-Targeting Mechanisms

Photosensitizers	Formulation	Average plasma $t_{1/2}$	Drug-light interval	Clinical status	Refs.
Verteporfin	Liposome	5–6 h (humans), 2–5 h (mice)	5–15 min	Approved for AMD	17, 22–24
Tin ethyletiopurpurin (SnET2)	Lipid emulsion	NA	10–45 min	Phase III trial	135–137
Lutetium texaphyrin (Lu-TeX)	Aqueous solution	~1 h (humans)	10–45 min	Phase I/II trial	138–141
Mono-L-aspartyl chlorin e6 (NPe6)	Aqueous solution	5–10 h (humans)	5–30 min	Phase I/II trial	142–147
ATX-S10(Na)	Aqueous solution	~45 min (rabbits)	Immediately–5 h	Preclinical	148–151
mTHPC (Foscan)	Lipid emulsion	~30 h (humans), ~7 h (mice)	5–180 min	Approved in EU for cancer	27–29, 152, 153
Tookad	Lipid emulsion	~20 min (humans)	0–30 min	Phase I/II trial	30–34
Hypericin	PEG/water	~1 h (mice)	30 min	Clinical trial in EU	154–157
MV6401	Lipid emulsion	~20 min (mice)	15 min	Preclinical	133, 158

Extending the drug-light interval to more than 50 minutes has been shown to decrease the therapeutic effect.²⁰ To extend the success of verteporfin, photosensitizers such as tin ethyletiopurpurin (SnET2, Purlytin) and lutetium texaphyrin (Lu-TeX, Optrin) are under clinical trial for AMD, on the basis of the same passive vascular-targeting principle.

In addition to its success in AMD treatment, passive vascular-targeting PDT is showing promise as a cancer treatment as well. Since AMD and cancer share almost the same vascular abnormalities, the photodynamic vascular-targeting modality that has been successful in AMD treatment should also have a role in cancer treatment, for which PDT was originally developed. Accumulative evidence has indicated that passive vascular-targeting PDT can be used for certain types of cancer treatment and is more effective in local tumor control than the traditional tumor cell-targeted PDT using the same photosensitizer and light doses but longer drug-light intervals. For instance, although verteporfin is largely used for AMD, we^{21,22} and others^{23,24} have shown that it can also be used for targeting neovasculature in tumors. As illustrated in Figure 2, fluorescence microscopic images indicate that verteporfin is predominantly localized within tumor vasculature at 15 minutes after administration. Light

irradiation at this time leads to considerable tumor destruction by inducing thrombosis formation and vascular shutdown, and this passive vascular-targeting PDT is more effective in tumor destruction than tumor cellular-targeting PDT using longer drug-light intervals.^{22–25}

Another example in this aspect is meso-tetrahydroxyphenylchlorin (mTHPC, Foscan), which has been approved in Europe for the treatment of head and neck cancer. Standard protocol employs a photosensitizer-light interval of about 4 days, so that an optimal tumor-to-normal tissue photosensitizer ratio can be obtained in order to target tumor cells with minimal normal tissue complications. However, experimental data on different tumor models demonstrates that the plasma drug level is a better predictor of tumor response than tumor photosensitizer concentration and that treatments using short drug-light intervals when plasma PS level is high produce much better results.^{26–29}

The most advanced tumor vascular-targeting photosensitizer based on the passive targeting principle is palladium-bacteriopheophorbide photosensitizer Tookad (WST09). Tookad is not water soluble and requires a Cremophor-based vehicle to make intravenous administration possible. The plasma half-life of Tookad is only about 20 minutes.³⁰ With such a fast plasma clearance, it is

necessary to give light almost at the same time or shortly after drug administration to induce an optimal vascular response.^{31–34} It has been shown that there is little PDT effect if light is given beyond 30 minutes after Tookad administration.³³ For such an application, intravenous infusion is preferred rather than bolus injection because the plasma drug concentration can be easily controlled by the infusion speed.³² Currently, Tookad is in a Phase I/II clinical trial for locally recurrent prostate cancer after radiation therapy.

Theoretically, any exogenous photosensitizer can be designed to target blood vessels based on this passive-targeting mechanism, as long as its pharmacokinetic properties enable it to remain sufficiently long enough in circulation. Then, the key is to find the optimal drug-light interval that entails predominant photosensitizer localization to the target vessels. For quite a few photosensitizers, a direct correlation between vascular effects and plasma photosensitizer concentration can be established. Illumination early after intravenous photosensitizer administration generally enhances vascular damage. In some cases, vascular

effects induced by PDT at a very short time after photosensitizer injection can be so strong that normal blood vessels are also affected, causing surrounding normal tissue damage. It has been documented that verteporfin-PDT using less than a 5-minute drug-light interval leads to significant choroidal and retinal damage resulting from the occlusion of normal blood vessels.^{35,36} PDT dosimetry can play an important role in situations where areas to be treated are delicately situated in close proximity to vital normal tissues. However simply adjusting the photosensitizer and/or light dose or prolonging the drug-light interval (to 15 minutes or more in the case of verteporfin) can make normal blood vessels essentially tolerant to PDT-induced vascular insult, whereas abnormal blood vessels are still sensitive to it, thereby avoiding normal tissue damage.

Passive photodynamic vascular targeting offers a simple means to selectively target blood vessels. This selectivity largely depends on the photosensitizer-light interval and the difference in response to vascular damage as a result of differences in structure and function between nor-

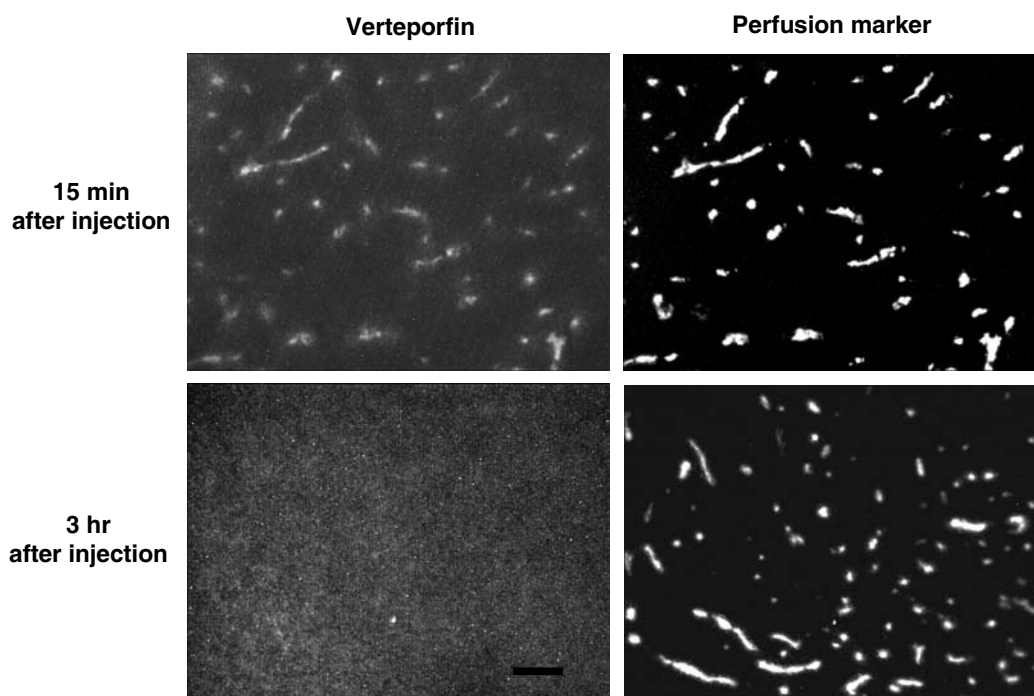


FIGURE 2. Fluorescence images of verteporfin in the subcutaneous MatLyLu rat prostate tumor model at 15 minutes and 3 hours after i.v. injection (1 mg/kg). The perfusion marker DiOC₇(3) (1 mg/kg, i.v.) was injected one minute before tumor excising to visualize the functional vasculature. The same microscopic field was imaged for both verteporfin and DiOC₇(3) by using appropriate filter sets for each dye. Bar, 100 μ m.

mal and abnormal neovascular blood vessels. In contrast to normal blood vessels, tumor vessels are leaky and stagnant in function, tortuous and dilated in morphology, and abnormal in structure (irregular pericytes and basement-membrane coverage).³⁷ As a result, tumor neovasculature has been shown to be more sensitive to photodynamic vascular-targeting therapy.³⁸ Despite the ability to spare normal blood vessels based on this difference in sensitivity to vascular damage, with a passive vascular-targeting strategy, both normal and abnormal blood vessels are likely exposed to the similar photosensitizer level when light is administered at a short time after drug injection. This suggests that exploration of active photodynamic vascular-targeting approaches, where the photosensitizer could be selectively confined to the neovascular components, would be useful.

B. Active Vascular-Targeting PDT

Active photodynamic vascular-targeting PDT relies on photosensitizer structural modification or a targeted drug delivery system so that the photosensitizing compound can be selectively bound to and retained in the targeted neovasculature components, to elicit a specific vascular effect (Fig. 1). In both cases, a targeting moiety that has a high affinity for neovasculature is directly linked to the photosensitizer molecules or to the photosensitizer delivery systems. The targeting moieties used in targeted drug delivery are peptides, antibodies, or other ligands that recognize molecules selectively expressed on newly formed blood vessels. Despite intensive efforts, a vascular marker that is truly specific to tumor vessels has not been identified.³⁹ Nevertheless, it has been found that some molecules show higher expression on tumor blood vessels than normal vessels, and these molecules can function as tumor vascular markers to achieve targeted delivery of therapeutic agents to tumor vasculature.

Various tumor vascular markers have been identified on endothelial cells, pericytes, and basement membranes.⁴⁰ Endothelial molecular markers are mainly surface membrane proteins overexpressed on tumor endothelial cells, which include growth factor receptors (e.g., VEGFR), integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$), CD105 (endoglin),

CD36 (thrombospondin-1 receptor), prostate-specific membrane antigen (PSMA), and tumor endothelial markers (TEMs). Many of these proteins have been used for imaging and targeting tumor neovasculature.^{40,41} Although quite a few markers, such as α -smooth muscle actin (α -SMA), platelet-derived growth factor receptor- β (PDGFR- β), and high-molecular-weight melanoma-associated antigen (NG2), have been found on pericytes, the expression of these markers is highly variable on pericytes and often significant on other types of cells as well. Thus, their application in targeted drug delivery remains to be determined. The vascular basement membrane is a self-assembled layer of proteins and proteoglycans formed by endothelial cells and pericytes.⁴² Its main components are type IV collagen, fibronectin, laminin, and heparin sulfate proteoglycan. As these components are necessary for angiogenesis and have been shown to be elevated in tumors, the basement membrane is a promising vascular target. For example, tumor fibronectin contains a distinctive extra-domain B (ED-B) that has been exploited for tumor angiogenesis imaging and targeted drug delivery.^{43,44}

Active vascular-targeting PDT is emerging as a promising modality for AMD and tumor treatments. Birchler et al. first reported the conjugation of photosensitizer tin (IV) chlorin e_6 with a human antibody fragment (L19) with high affinity for the ED-B domain of fibronectin.⁴⁵ In a rabbit cornea angiogenesis model, L19 antibody selectively recognized newly formed blood vessels, but not pre-existing vessels. The conjugate was shown to selectively cause coagulation in corneal neovasculature but not in blood vessels of the surrounding normal tissue when light is delivered 8 hours after administration. At that time point, there was little amount of photosensitizer conjugate (<1%) remaining in the circulation, suggesting that the contribution of passive vascular damage is limited. To target the overexpression of VEGFR on the membranes of angiogenic endothelial cells, Renno et al. conjugated verteporfin (after isolation from a liposomal formulation) to VEGFR-2-binding peptide Ala-Thr-Trp-Leu-Pro-Pro-Arg (ATWLPPR) via a polyvinyl alcohol polymer (PVA) linker.⁴⁶ The conjugate displayed similar photophysical properties and photosensitizing activity as verteporfin. PDT us-

ing this targeted verteporfin with a 1 hour drug-light interval was found to be more effective in CNV closure, with less significant damage in normal tissue than standard nontargeted verteporfin in a rat CNV model. If this promising result can be confirmed in the clinical setting, it will greatly improve the effectiveness and safety of current verteporfin therapy for AMD.

Although the ATWLPPR peptide is traditionally considered and used as a VEGFR-2-specific peptide, recent evidence demonstrates that it actually binds to neuropilin-1 (NRP-1) rather than VEGFR-2.⁴⁷ NRP-1 is also a type of VEGF receptor and is overexpressed on tumor endothelial cells as well as tumor cells.⁴⁸ Tirand et al. recently conjugated a chlorin photosensitizer to the ATWLPPR peptide via a 6-aminohexanoic acid spacer.⁴⁹ Their results indicated that ATWLPPR and its photosensitizer conjugate bind exclusively to NRP-1 rather than VEGFR-2. The intracellular concentration of conjugate in the human umbilical vein endothelial cells (HUVECs) was up to 25 times higher than for the free photosensitizer. Furthermore, the addition of ATWLPPR is able to inhibit cellular drug uptake, suggesting a specific receptor-mediated pathway. In agreement with the drug accumulation data, photocytotoxicity of the conjugate was more than 10 times more potent than the free drug in HUVECs. The conjugate seemed stable in the circulation, with an average half-life of 10–13 hours and reached a peak tumor drug level at 1 hour after administration in the U87 human glioma xenograft. Unfortunately, *in vivo* PDT activity was not reported in the study.

In addition to photosensitizer molecules, photosensitizer delivery systems can also be modified to actively target neovasculature. Ichikawa et al. encapsulated BPD-MA in a polyethylene glycol (PEG)-modified liposome designed to remain in the circulation for a long time and linked it to a peptide (Ala-Pro-Arg-Pro-Gly, APRPG) that binds specifically to angiogenic vessels.⁵⁰ Tumor uptake of the APRPG-PEG-modified liposome was about 4-fold higher than the untargeted PEG-liposome 3 hours after administration in a mouse Meth-A sarcoma tumor model. Interestingly, untargeted PEGylated BPD-MA liposomes had little photodynamic activity, presumably due to poor

intracellular uptake whereas APRPG-PEG-liposomes showed a significant PDT effect. This result highlights the importance of using vascular homing molecules to achieve intracellular delivery of a photosensitizing agent. Solely increasing tumor drug uptake does not necessarily translate into increase in activity.

Despite these promising results, some key issues are not adequately addressed in these studies. For instance, the plasma stability of these conjugates, the interaction with vascular components, and the pharmacokinetics in plasma and tumor tissue are not well characterized. This information is necessary for determining whether the conjugate is able to specifically target tumor vasculature and what the optimal condition is for targeting tumor vessels. Ideally, the conjugate should have a strong affinity to the neovasculature and fast plasma clearance so that light can be delivered at an optimal time period when only the neovascular structure has significant photosensitizer uptake. Illumination prior to this optimal time period is likely to increase the contribution of nonspecific vascular effect because the photosensitizer plasma concentration remains high. Light treatment beyond after the optimal period might lead to reduced damage to the vascular compartment and increased damage to the parenchyma cells because conjugate molecules may leak into the tissue parenchyma compartment.

IV. TUMOR CELL TARGETING WITH PDT

Tumor cells are obvious and legitimate targets of PDT. A long-cherished goal of any cancer therapy is to kill tumor cells without much involvement of the normal cells. For PDT, selective tumor cell targeting can be achieved by at least two principles. One is the specific light delivery provided by recent developments in various laser light sources and fiber optic delivery devices.^{51,52} The other is the targeted delivery of photosensitizers, which is based on the passive or active targeting principle, as illustrated in Figure 3. Additionally, it has been reported that the connective tissue is insensitive to PDT damage, and that normal tissue healing over remaining tissue scaffolds after PDT is quite good.^{53–55} These features make PDT a good candidate for tumor cell targeting.

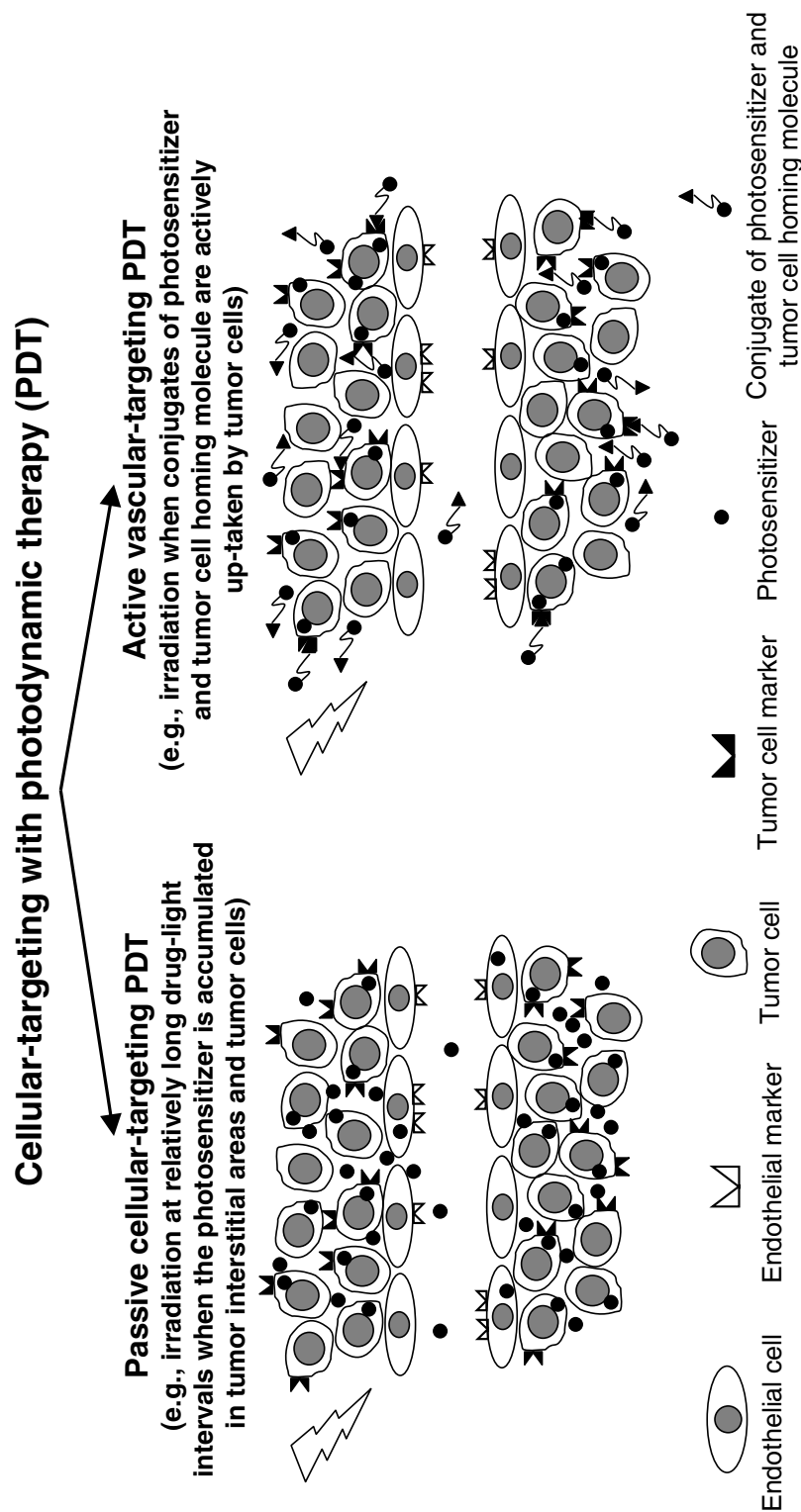


FIGURE 3. Cellular-targeting with photodynamic therapy (PDT). Depending on whether a tumor cellular-targeting molecule is used in the preparation of photosensitizers, cellular-targeting PDT can be divided into passive and active cellular-targeting PDT. Passive cellular-targeting PDT depends on the photosensitizer physicochemical properties and tumor tissue pathophysiological conditions (such as EPR effect) to deliver photosensitizing agents to tumor cells. To allow adequate photosensitizers to accumulate in tumor cellular compartment, light treatment is generally given at hours or days after photosensitizer administration. In active cellular-targeting PDT, photosensitizers or photosensitizer drug delivery systems are usually modified with a tumor cellular-targeting molecule so that only tumor cells expressing the targeting molecules will have significant uptake of photosensitizing agents and be targeted by PDT.

A. Passive Cellular-Targeting PDT

Passive cellular-targeting PDT depends on the photosensitizer physicochemical properties and tumor tissue pathophysiological conditions to deliver photosensitizing agents to tumor cells (Fig. 3). As a matter of fact, most of the past efforts in photosensitizer development rely on finding naturally occurring compounds that appear promising in selective tumor cell damage. Although all of the current photosensitizers do not selectively accumulate in tumor tissues, tumor tissues typically display several times more photosensitizer uptake than the surrounding skin and muscle tissues in most of the preclinical studies.⁸ This relative preferential tumor uptake is primarily attributed to photosensitizer physicochemical properties and tumor pathophysiological conditions, which provide favorable conditions for the preferential accumulation of macromolecular drugs in tumor tissues. Unlike normal blood vessels, tumor (angiogenic) blood vessels are generally more permeable to circulating molecules, allowing more drugs to extravasate into the tumor interstitial space. The impaired lymphatic drainage in tumor tissues further retards the clearance of macromolecular drugs from the tumor interstitial area. Through this enhanced permeability and retention (EPR) effect, therapeutic agents are able to accumulate in tumor tissues.⁵⁶ To passively target tumor cells based on this EPR effect, the therapeutic agents or formulations should be macromolecules (above 40 kDa) and preferably have long circulation times because tumor drug uptake has been shown to be proportional to the drug circulation time. Although most photosensitizers are small molecules, they are generally bound to plasma proteins in circulation and behave like macromolecules. Thus, preferential photosensitizer tumor accumulation can occur through the EPR effect.

Once photosensitizers are extravasated into the tumor interstitial space, they need to be associated with tumor cell membranes or internalized into tumor cells to generate photocytotoxicity. Photosensitizer physicochemical properties and plasma protein-binding behavior play an important role in determining how and to what extent tumor cells uptake photosensitizers. For example, as most hydrophobic photosensitizers tend to bind to LDL,

they may enter the cell via LDL receptor-mediated endocytosis mechanisms.⁵⁷ Hydrophobic photosensitizers are more likely to be associated with LDL and lead to an increased intracellular uptake through this pathway.⁵⁸ Hydrophobic photosensitizer molecules can also be released from the photosensitizer-plasma protein complex in tumor interstitial areas and passively diffuse into tumor cells.⁵⁹ More hydrophilic photosensitizers, such as ATX-S10 and NPe6, are likely to bind to albumin and HDL and be taken up by tumor cells via the nonspecific endocytosis pathway.^{60–62}

Intracellular localization of photosensitizers is not as confined as originally thought. They can be co-localized in different cell compartments, such as cell membranes, mitochondria, lysosomes, and endoplasmic reticulum. Thus, tumor cell responses to PDT are complicated and dependent on many factors, such as the photosensitizer, PDT conditions, and the type of tumor cell/model. Almeida et al. have summarized various intracellular signaling pathways following photosensitization treatment.⁶³ Since the goal of PDT is to induce tumor cell death usually occurring by apoptosis or necrosis, cell death pathways (especially apoptotic cell death) have been extensively studied. PDT can induce tumor cell apoptosis via mitochondria or death receptor-mediated pathways. At high/lethal PDT doses (no limit in photosensitizer concentration, light dose, and oxygen), these cell death signaling pathways will be immediately executed, leading to tumor cell death.⁶⁴ However, at low/sublethal PDT doses (usually the case in PDT treatments), tumor cell death will be delayed and dependent on a balance between cell death signaling and PDT-induced cell survival signaling. The cell survival signal can come from the innate cellular protective response toward oxidative damage and apoptotic death signaling (e.g., expression of heat-shock protein, antioxidant enzymes, and antiapoptotic proteins) and response to PDT-induced tissue damage (e.g., expression of hypoxia-inducible factor-1 α [HIF-1] and vascular endothelial growth factor [VEGF] in response to tumor hypoxia). Inhibition of these survival signals has been shown to enhance PDT effects, both *in vitro*⁶³ and *in vivo*.⁶⁵

To make passive cellular-targeting PDT effective, light is often given at a relatively long time after photosensitizer injection. The use of

this long drug-light interval is necessary for photosensitizer accumulation in the tumor cellular compartment. As shown in Figure 2, verteporfin has distributed into the tumor tissue at 3 hours after administration, in contrast to the intravascular localization at a 15-minute time point. PDT with long drug-light intervals induces phototoxicity to tumor cells via a passive targeting mechanism. However, passive vascular targeting due to the existence of photosensitizer molecules remaining in tumor vasculature might also contribute to the final PDT outcome. The relative contribution of vascular- versus tumor-cellular effects is dependent upon the distribution of photosensitizing agents in two compartments, which is governed by photosensitizer affinity to each compartment and its pharmacokinetic properties.

B. Active Cellular-Targeting PDT

As passive cellular-targeting PDT, based on innate photosensitizer physicochemical and tumor pathophysiological properties, is often not able to selectively kill tumor cells, active cellular-targeting PDT employing specific modifications of photosensitizers or photosensitizer carrier systems with molecules possessing a high affinity for various specific tumor markers has been actively pursued. Progress in cancer cell and molecular biology has resulted in the discovery of many cellular and molecular targets that can be exploited for targeted drug delivery. Modification of existing photosensitizing agents with tumor cell-targeting molecules intends to restrict photodynamic action to the targeted cells by altering photosensitizer pharmacokinetics and biodistribution. As a result, PDT efficacy is greatly increased, whereas phototoxicity is significantly reduced. Active cellular-targeting PDT has been explored to target not only a variety of surface proteins/peptides and receptors that are overexpressed on the tumor cell membrane, but also tumor cell nuclei, where the uptake of photosensitizers is generally low.

1. Tumor Surface Proteins or Peptides

Mew et al. first introduced the term photoimmunotherapy to describe the use of photosensi-

tizer haematoporphyrin-antibody conjugates designed to specifically target tumor tissues.^{66,67} Haematoporphyrin was conjugated directly to antibodies against DBA/2J mouse M-1 myosarcoma or human leukemia-associated antigen (CAMAL) using the carbodiimide procedure. Although the conjugate stability, antigen binding, and biodistribution were not rigorously tested based on current standards, photoimmunoconjugates were indeed shown to induce selective tumor growth inhibition. Promising results of direct photosensitizer and antibody conjugation were also reported by Pogrebniak et al.⁶⁸ They conjugated hematoporphyrin to a MAb 45-2D9 recognizing a cell-surface glycoprotein on *ras* oncogene-transformed NIH 3T3 cells and demonstrated both in vitro and in vivo that the photoimmunoconjugate was able to kill tumor cells more selectively and effectively than the free photosensitizer.

The indirect linkage of multiple photosensitizer molecules to antibodies via backbone molecules can produce more reproducible and quantifiable conjugates and, more importantly, retain the binding affinity and biological activity of the antibody and photosensitizer. Chlorin e6 was conjugated to an anti-T-cell monoclonal antibody through dextran or polyglutamic acid (PGA) linker to target human T leukemia cells (HBP-ALL).^{69,70} The conjugate with a chlorin/antibody molar ratio of more than 30 still retains about 80% of the antibody-binding affinity and displays the same absorption spectrum and singlet oxygen quantum efficiency as free chlorin e6. Another chlorine photosensitizer, BPD, was covalently linked to antibody MAb 5E8 targeting a cell-surface glycoprotein on human lung squamous cell carcinomas via a polyvinyl alcohol (PVA) linker.^{71,72} The BPD-PVA-MAb 5E8 conjugate exhibited more selective and enhanced phototoxicity over free BPD and a control conjugate with an irrelevant antibody in A549 human lung cancer cells. The biodistribution of the conjugate was also studied in A549 xenograft following i.v. injection and compared to free BPD and the control conjugate.⁷³ Both conjugates have longer circulation and tissue retention than free BPD. Although significant uptake of the BPD-PVA-MAb 5E8 conjugate was observed in the lung, liver, and spleen reticuloendothelial system (RES), it reached the highest tumor-drug concentration

at 14 hours after injection, which is higher than the peak tumor levels of control BPD conjugate and free BPD that occurred at 3 hours after administration. These early studies not only laid the foundation of photoimmunotherapy in cancer cell targeting, but also showed its promise for subsequent cancer therapy development.

The functional carboxyl groups of photosensitizer chlorin e6 can be readily conjugated with targeting molecules. Thus, this type of photosensitizer is often used in the conjugation study. Both chlorin e6 and chlorin e6 monoethylene-diamine monoamide (CMA) were conjugated to two antibodies: OC125 recognizing an ovarian carcinoma glycoprotein antigen CA 125 and an anticolon cancer monoclonal antibody 17.1A. Different linkers were used, and the systemic biodistribution and photoactivity of the immunoconjugate were evaluated. An immunoconjugate between CMA and OC125 via polyglutamic acid linkage was demonstrated by ELISA assay to retain significant antigen-binding affinity and specificity.⁷⁴ Phototoxicity tested on ascites or pleural fluid cells from patients with ovarian or nonovarian cancers indicated that the conjugate was significantly more cytotoxic to ovarian cancer cells than nonovarian cancer cells. The conjugate was further evaluated in an ascitic ovarian cancer model in the Balb/c nude mice induced by intraperitoneal (i.p.) injection of NIH:OVCAR3 human ovarian cancer cells.⁷⁵ Although both the CMA-OC125 immunoconjugate and free CMA reached peak tumor concentrations at 24 hours following i.p. injection, tumor uptake of CMA-OC125 was about 3-fold higher than for the free drug. Tumor levels of the conjugate were, on average, about 6-fold higher than normal tissues, such as skin, blood, and liver, at 23 hours after administration. PDT using the conjugate was effective in killing ascitic tumor cells in a dose-dependent manner, but at high doses induced significant normal tissue damage and even death. A protocol of multiple low-dose PDT treatments turned out to be an effective and safe regimen for reducing tumor burden.^{75,76}

A photoimmunoconjugate consisting of chlorin e6 linked via positively charged poly-L-lysine to the F(ab')₂ fragment of antibody OC125 was used to study the effect of charge modification on photosensitizer conjugate uptake and

phototoxicity.⁷⁷ The poly-L-lysine and chlorin e6 complex was first polysuccinylated and then covalently conjugated to the antibody to produce a negatively charged conjugate. Both the positive and negatively charged immunoconjugates still preserved antigen-binding affinity, as suggested by competitive inhibition with the innate antibody in a human ovarian cancer cell line NIH-OVCAR-5. However, the cellular uptake of the positively charged conjugate was much higher than the negatively charged conjugate and free chlorin e6, possibly due to enhanced internalization. In the NIH-OVCAR-5 human ovarian tumor xenograft, the i.p.-administered positively charged immunoconjugate delivered much higher amounts of chlorin e6 to the tumor tissue than the negatively charged immunoconjugate and a nontargeted immunoconjugate prepared with a nonspecific IgG and free chlorin e6.⁷⁸ Multiple intraperitoneal PDT performed at 3 hours after photosensitizer administration (i.p.) in the same tumor model demonstrated that tumor-bearing animals tolerated the repeated PDT treatments well, and tumor responses (residual tumor weight and animal survival time) to the positively charged conjugate were much better than responses to the negatively charged or free chlorin e6.⁷⁹ The same strategy was employed to target colorectal cancer cells. Two charged photoimmunoconjugates of chlorin e6 and an anticolon cancer monoclonal antibody 17.1A were prepared and found to have selective photocytotoxicity to antigen-positive cells, as compared to the nonspecific IgG conjugate.⁸⁰ The positively charged conjugate delivered over 4 times more chlorin e6 to tumor cells than the negatively charged one and was significantly more photoactive than the negatively charged conjugate and free chlorin e6. These results suggest that the positive charge improves endocytosis and subsequent lysosomal degradation of the immunoconjugate.

However, this is not always the case. The same chlorin e6 negatively charged immunoconjugate administered via i.v. injection had a higher tumor accumulation and tumor-to-normal tissue ratio than the positively charged one in a colorectal cancer-induced hepatic tumor model in nude mice.⁸¹ Using an interstitial fiber, hepatic tumors were treated with light at 3 hours after i.v. injection of either negatively charged conjugates

or free chlorin e6, for comparison.⁸² PDT with negatively charged immunoconjugates was highly effective in reducing tumor weight and prolonging animal survival, whereas free chlorin e6 PDT was ineffective. These data, seemingly contradictory to the previous results of OC125 conjugates, highlight the importance of target-cell location, histological type, and administration route in affecting photoimmunoconjugate uptake and photoimmunotherapy efficacy.

Several studies have been reported to actively target superficial skin cancers using photosensitizer conjugates. A series of immunoconjugates consisting of photosensitizer tin(IV) chlorin e6 (SnCe6) and antimelanoma antibody MAb 2.1 linked via dextran were prepared to target malignant melanoma cells. The conjugation involved the site-specific modification of the antibody oligosaccharides with a single chain-terminal hydrazide group, which is the coupling point between dextran and the antibody.^{83,84} Conjugates with varied photosensitizer-to-antibody molar ratios (up to 18.9) were prepared. A competitive inhibition radioimmunoassay demonstrated that the conjugate retained a good antigen-binding activity, similar to the native antibody. Clonogenic assay showed that the conjugate was selectively phototoxic to the antigen-presenting SK-MEL-2 human malignant melanoma cells. However, it was noted that the quantum yield of singlet oxygen generated by the conjugated SnCe6 was significantly less than that observed with the free drug as a result of reduced triplet yield, which might suggest the formation of aggregates.⁸⁵ Chlorin e6 monoethylenediamine monoamide (CMA) was also conjugated to a melanoma-reactive monoclonal antibody IG12.⁸⁶ The immunoconjugate was about 9 times more phototoxic toward the targeted OCM431 human uveal melanoma cells than the nontargeted RPMI1846 melanoma cells, whereas the free photosensitizer was more than 2-fold less phototoxic than the conjugates and, importantly, did not possess selective phototoxicity.

Photosensitizer aluminum tetrasulfophthalocyanine (AlPcS₄) was covalently coupled to a MAb 35A7 against carcinoembryonic antigen (CEA) via a five-carbon spacer chain.⁸⁷ Conjugates with AlPcS₄ to mAb 35A7 molar ratios of 5 to 16 were prepared and evaluated for targeting T380 hu-

man colon carcinoma xenografts in nude mice. A significant finding of this study was that conjugation with photosensitizer via a five-carbon spacer led to no adverse effect on antibody biological activity, as shown by antigen-binding assay and tumor distribution examination. The conjugates were phototoxic to LoVo colon carcinoma cells. However, in vivo phototoxicity of these conjugates was not reported, making it impossible to evaluate the in vivo activity.

2. Growth Factor Receptors

One of the hallmarks of cancer is uncontrolled cell growth, which can be partially attributed to the overexpression of various growth factor receptors.⁸⁸ Among these, the tumorigenic functions of the epidermal growth factor receptor (EGFR) and HER-2 receptor have been most extensively studied and, consequently, actively pursued for therapeutic targeting. It is not surprising to note that many tumor cellular-targeting photosensitizer conjugates are developed to target growth factor receptors, especially EGFR and HER-2 receptor.

Conjugation of EGFR antibody C225 to chlorin e6 yielded a photoimmunoconjugate designed to target EGFR-overexpressing tumor cells in the hamster cheek pouch carcinogenesis model.⁸⁹ To diagnose malignancy and monitor treatment response to targeted PDT with chlorin e6-C225, a near-infrared dye Cy5.5 was also conjugated to C225. The results demonstrated that Cy5.5-C225 was able to diagnostically delineate tumor regions and prognostically indicate tumor response to EGFR-targeted PDT. Since EGF is an endogenous ligand of EGFR, Gijssens et al. conjugated EGF and photosensitizer Sn-(IV) chlorin e6 (SnCe6) via three different carriers—dextran (Dex), human serum albumin (HSA), and polyvinylalcohol (PVA)—to target EGFR expressing tumor cells.^{90,91} As a comparison, SnCe6 was also conjugated to carrier molecules, only without EGF targeting. Although EGF-PVA-SnCe6 conjugate exhibited a higher photocytotoxicity (IC₅₀: 2.8 microM) than EGF-Dex-SnCe6 (IC₅₀: >10 microM) and free SnCe6 (IC₅₀: >10 microM) in A431 cells, nontargeted PVA-SnCe6 conjugates showed a similar photocyto-

toxicity (IC_{50} : 3.5 μ M) to EGF-PVA-SnCe6, suggesting that EGF does not play a major role in conjugate uptake.⁹⁰ The EGF-Dex-SnCe6 conjugates have better cellular uptake than EGF-PVA-SnCe6. However, EGF-Dex-SnCe6 only displayed a slight increase in photocytotoxicity over Dex-SnCe6, again indicating a limited EGF receptor-mediated active uptake. The EGF-HSA-SnCe6 conjugates possess the highest cellular uptake and photocytotoxicity (IC_{50} : 63 nM), which can be competitively inhibited by free EGF.⁹¹ These results clearly demonstrate that the efficiency of photoimmunoconjugates strongly depends on the carrier molecules.

EGFR-targeting photosensitizer immunoconjugates were prepared by coupling BPD to an EGFR antibody C225.⁹² To increase the conjugate solubility and prevent the formation of aggregates, a small number of antibody lysines (<3 per antibody) were first PEGylated with a 10-kDa branched polyethylene glycol (PEG). BPD, dissolved in a 50% dimethyl sulfoxide-50% aqueous two-solvent system, was covalently linked to the remaining antibody lysines. The resultant conjugates were shown to maintain antigen-binding activity and have low nonspecific macrophage uptake. The BPD-C225 conjugates induced photocytotoxicity in EGFR-overexpressing A-431 cells but had no significant effect on EGFR-negative NR6 cells. Further in vitro photobiological evaluation of this conjugate indicated that BPD-C225 immunoconjugates were more selective, but less effective, than free BPD in killing EGFR-overexpressing cells.⁹³ The promise of EGFR-targeted photoimmunotherapy based on the conjugation of EGFR antibody and BPD was even extended to in vivo studies. Hemming et al. covalently conjugated an EGFR antibody to BPD via a PVA linker and evaluated its biodistribution and PDT efficacy in the hamster cheek pouch model of squamous cell carcinoma.⁹⁴ The EGFR-targeted BPD conjugate demonstrated excellent tumor distribution selectivity. The tumor-to-normal tissue ratio of photosensitizer level was 26 for the BPD-EGFR antibody conjugate and only 2 for free BPD. Tumor response to PDT was consistent with the distribution result. Animals treated with free BPD had a 1-month tumor-free survival of 67%, whereas animals treated with the tumor-specific BPD-EGFR antibody conjugate

at one twentieth the total dose of free BPD had an 80% 1-month tumor-free survival. This in vivo study clearly demonstrated that photosensitizer distribution and therapeutic efficacy could be greatly improved through the conjugation to a tumor-specific antibody. The increased efficacy might come from both PDT-induced photocytotoxicity and antibody-induced cytotoxicity.

Both internalizing and noninternalizing antibodies were conjugated to a chlorin photosensitizer mTHPC to determine which conjugate generated better phototoxicity.⁹⁵ Photosensitizer mTHPC was first tetracarboxymethylated to increase water solubility and create functional groups for the subsequent conjugation to antibody lysine residues. The modified mTHPC was covalently conjugated to a noninternalizing anti-CD44v6 MAb U36 or an internalizing anti-EGFR MAb 425 to target head and neck squamous cell carcinoma. The conjugates with a mTHPC:MAb molar ratio of up to 4 were shown to be stable, immunoreactive, and photoactive in vitro. In a head and neck carcinoma HNX-OE xenograft, immunoconjugates had better tumor selective distribution than free mTHPC. An important finding of this study was that photosensitizer coupled to an internalizing MAb displayed more phototoxicity than that conjugated to a noninternalizing MAb. Similar results were also reported in studies comparing aluminium tetrasulphthalocyanine (AlPcS₄) immunoconjugates with a noninternalizing MAb 35A7 recognizing carcinoembryonic antigen (CEA) and an internalizing HER2 MAb FSP 77 and 17.1A.^{96,97}

This hypothesis was further tested with two hydrophilic photosensitizers.^{98,99} The rationale for using hydrophilic photosensitizers is obvious, because this type of photosensitizer generally has low photocytotoxicity due to low cell membrane affinity, and they are good candidates for photoimmunoconjugation because of good water solubility. The question being addressed was whether conjugating hydrophilic photosensitizers to an internalizing MAb would enhance the phototoxicity. Hydrophilic photosensitizer TrisMPyP- ϕ CO₂H⁹⁸ or aluminum (III) phthalocyanine tetrasulfonate [AlPc(SO₃H)₄]⁹⁹ was coupled to internalizing antibodies (MAbs U36 and 425) or noninternalizing antibody E48 against a glycosylphosphatidylinositol-anchored surface antigen.

Similar to their previous mTHPC conjugates, new photoimmunoconjugates were stable and showed good immunoreactivity as long as the photosensitizer:MAB ratio was maintained below 4 in both cases. The conjugates with a low photosensitizer:MAB ratio demonstrated selective tumor accumulation in head and neck carcinoma HNX-OE xenografts, although conjugate tumor uptake was always lower than native antibodies. At higher molar ratios, the solubility of conjugates was significantly decreased, which led to a faster plasma clearance and lower tumor uptake. In A431 cells, conjugates with an internalizing antibody (MAB U36 or 425) were significantly more phototoxic than conjugates with a noninternalizing MAB E48 and free photosensitizers, which showed a limited or no photocytotoxicity at all. For instance, AlPc(SO₃H)₄-MAB 425 conjugate was about 7500 times more toxic to A431 cells than the free sensitizer (IC₅₀, 0.12 nM vs. 900 nM). However, in a subsequent study involving more cell lines, phototoxicity of AlPcS₄-MAB was found to only correlate strongly with the total cell-binding capacity (both internalized and cell-surface bound) and not with the internalization capacity only.¹⁰⁰ Thus, the selection of internalizing or noninternalizing antibody for photoimmunotherapy is not that straightforward. Photosensitizer physicochemical properties, antibody-binding affinity, antigen expression, method of conjugation, and the type of targeting cell are important factors that should be considered.

Almost all the photoimmunoconjugates developed so far are to target a single epitope on the target cells because the monoclonal antibodies used for the conjugation can only bind to and target a single epitope. It was recently shown that the combination of immunoconjugates targeting different epitopes is better than a single immunoconjugate therapy.¹⁰¹ Photosensitizer pyropheophorbide-a (PPa) was covalently conjugated to either HER55 or HER66, both of which were anti-HER2 monoclonal antibodies. Similar to previous BPD-C225 conjugates constructed in the same manner,^{92,93} HER2-targeting immunoconjugates were more selective, but less effective, than the free photosensitizer in killing HER2-overexpressing cells, suggesting quenching of photoimmunoconjugates and possible changes in intracellular localization. Nevertheless, this study clearly demonstrated that

multi-epitope targeting with a combination of HER55 and HER66 pyropheophorbide-a conjugates was significantly more effective than single epitope photodynamic targeting with a single anti-HER2 immunoconjugate.

3. LDL Receptors

LDL receptors are a group of cell surface receptors that transport physiological molecules (e.g., cholesterol), drugs, and drug formulations into cells through a receptor-mediated endocytosis pathway.¹⁰² This process involves receptor recognition of a ligand, internalization through clathrin-coated pits, and degradation following fusion with lysosomes. Apolipoprotein B-100 (ApoB-100) on the outer shell of LDL is one of LDL receptor ligands and is responsible for the recognition and binding of LDL to LDL receptors. Although their expressions are somewhat elevated in tumor cells due to increased cell membrane synthesis, this targeting approach is not very specific because LDL receptors are actually expressed on almost all types of cells, especially cells in the liver and adrenal gland. Nevertheless, LDL receptors have been shown to be important in intracellular delivery of hydrophobic photosensitizers, and substantial efforts have focused on modifying photosensitizer structures to increase the interaction between photosensitizer conjugates and LDL receptors so that more drugs can be actively transported into tumor cells.

A seemingly straightforward approach is to covalently conjugate photosensitizer molecules to LDL to actively target LDL receptors. LDL receptor-mediated endocytosis appears to be involved in the cellular uptake of LDL-photosensitizer conjugates. But the extent of active cellular uptake is highly variable, dependent upon the photosensitizer, conjugation chemistry, and the target cells. For example, when haematoporphyrin (HP) is conjugated to LDL, its uptake is increased in the LDL receptor upregulated NIH3T3 cell line and inhibited in the presence of very high levels of free LDL.¹⁰³ However, the uptake of HP-LDL conjugate is even more significant in J774.2 macrophages. This result, together with the observation that HP-LDL conjugates form aggregates, suggests that chemical preparation likely affects LDL

ApoB-100 function. Therefore, cells possessing scavenger receptors and/or phagocytic activity mainly take up the conjugate rather than tumor cells expressing LDL receptors. Chlorin e6 (Ce6) was covalently conjugated to LDL via the carbodiimide activation method,¹⁰⁴ where the Ce6-LDL conjugate had a significantly higher (4-5 times) cellular uptake than free Ce6 and Ce6 noncovalently complexed with LDL. Although nonspecific association did occur, an active receptor-mediated uptake pathway was clearly demonstrated by receptor saturation and competitive inhibition experiments. Phototoxicity induced by the Ce6-LDL conjugate was more than 8 times higher than free and LDL-mixed Ce6, which demonstrates the importance of choosing the type of photosensitizer and conjugation method in influencing PDT-targeting capability.

An alternative approach to targeting LDL receptors is to improve photosensitizer incorporation into LDL, which can be achieved chemically by modifying photosensitizer physicochemical properties and/or pharmaceutically formulating photosensitizers in drug delivery systems. Photosensitizer tetrasulfonated aluminum phthalocyanine (AlPcS₄) was modified with alkyl chains of various length.⁵⁸ Intracellular uptake of the AlPcS₄ derivatives depended on the alkyl chain length, where alkylation of AlPcS₄ with long chains increased uptake more than short chains: AlPcS₄(C16) > AlPcS₄(C12) > AlPcS₄(C8) > AlPcS₄(C4). Human LDL inhibited cellular uptake of alkyl chain conjugates, suggesting an active LDL receptor-mediated pathway. The photocytotoxicities of AlPcS₄ derivatives correlated with the intracellular uptake results, demonstrating that modifying photosensitizer molecules with a long hydrophobic chain facilitates conjugate insertion into the lipid core of the LDL particles. Interestingly, Zheng and colleagues reconstructed LDL and used it as an endogenous delivery vehicle to achieve targeted delivery of photosensitizers. They synthesized two photosensitizer conjugates—a pyropheophorbide cholesterol oleate conjugate¹⁰⁵ and a tetra-*t*-butyl silicon phthalocyanine bearing two oleate moieties at its axial positions¹⁰⁶—that could be reconstituted into the LDL lipid core with a very high payload (up to several thousand photosensitizer molecules per LDL molecule). Importantly, the reconstituted LDL with such a high

photosensitizer payload retained the mean size of native LDL and could be internalized into human hepatoblastoma G2 (HepG2) cells via LDL receptors. As a result, photocytotoxicity of reconstituted LDL loaded with photosensitizers was significantly higher than the free photosensitizers, which presents a unique way to deliver large amounts of photosensitizer molecules to tumor cells.

4. Transferrin Receptors

Transferrin receptors are cell membrane receptors overexpressed on certain cancer cells due to increased cancer cell proliferation.¹⁰⁷ Transferrin (molecular weight, 80,000), being an endogenous ligand to transferrin receptors, is a major protein in the circulation involved in iron transportation. After transferrin binds to the receptor, the transferring iron-receptor complex is internalized and the iron is released intracellularly. Because of its high affinity for the transferrin receptor, transferrin has been used as a ligand to deliver anticancer drugs, including photosensitizers, via receptor-mediated endocytosis.

Haematoporphyrin was conjugated covalently to transferrin using an *N*-hydroxysuccinimide ester linkage.¹⁰⁸ Although the fluorescence of the conjugate was quenched, the conjugate had a similar singlet oxygen quantum yield to the free porphyrin. The uptake of the hematoporphyrin-transferrin conjugate in NIH 3T3 and HT29 cells was somewhat dependent upon receptor-mediated endocytosis, as indicated by partial inhibition by free transferrin and increased uptake following transferring-receptor upregulation. Transferrin was also covalently coupled to chlorin e6 using a procedure involving protein binding to quaternary amino-bearing sephadex prior to chlorin e6 modification to maintain transferrin activity.¹⁰⁹ Although the transferrin-chlorin e6 conjugate had about 70% efficiency of singlet oxygen yield compared to free chlorin e6, it was over 10 times more phototoxic than free chlorin e6 in human MCF7 and rat MTLn3 mammary adenocarcinoma cells.

Hydrophilic photosensitizer AlPcS₄ was also encapsulated into transferrin-conjugated PEG liposomes to target transferrin receptor-overexpressing tumor cells.¹¹⁰ Internalization of trans-

ferrin-AIPcS₄ liposomes was shown to involve transferrin receptors, leading to a high intracellular concentration in HeLa cells that overexpress transferrin receptors. As a result, the phototoxicity of the transferrin-AIPcS₄ liposome was about 10 times higher than free AIPcS₄, whereas a non-targeted AIPcS₄ liposome was not phototoxic at all. Further studies revealed that transferrin receptor-overexpressing human AY-27 cancer cells incubated with transferrin-AIPcS₄ liposome had more than a 100-fold higher intracellular AIPcS₄ concentration than cells incubated with non-targeted liposomes.¹¹¹ In an orthotopic AY-27 bladder tumor model, intravesical instillation of the targeted liposome resulted in tumor uptake that was 18 and 78 times higher than normal urothelium and submucosa/muscle, respectively. Although instillation of free AIPcS₄ results in nonselective accumulation throughout the whole bladder wall, nontargeted liposome instillation produced no tissue-PS accumulation. The selective uptake of transferrin-AIPcS₄ liposome led to a greater phototoxicity to tumor cells, which clearly demonstrates that transferrin-guided photosensitizer conjugates can be used to selectively target transferrin receptor-overexpressing tumors.

This approach, however, does not work for all photosensitizers. An attempt to encapsulate the hydrophobic photosensitizer hypericin within transferrin-conjugated liposomes to target HeLa cells did not significantly improve intracellular hypericin accumulation.¹¹² A drug-embedding stability study revealed that this hypericin-loaded liposome was not stable. Hypericin, because of its hydrophobic nature, was mainly integrated between lipid bilayers of the liposome and could thus rapidly leak out to redistribute to plasma proteins. In this way, the liposome formulation of hypericin behaved more like the free drug, whereas the more hydrophilic AIPcS₄ was stably confined within the liposomal core.

5. Folic Acid Receptor

Folic acid receptors are membrane receptors responsible for the uptake of folic acid, a vitamin essential for *de novo* nucleotide synthesis, via receptor-mediated endocytosis.¹¹³ Folate receptors are only expressed on certain epithelial cells and

overexpressed on epithelial malignant cells such as breast, ovary, brain, and lung cancers. Therefore, these receptors can be explored for targeted drug delivery. Two folic acid conjugates were recently prepared using photosensitizer 4-carboxyphenylporphyrine and two different linkers—hexane-1,6-diamine and 2,2'-(ethylenedioxy)-bis-ethylamine.¹¹⁴ Both conjugates demonstrated a nearly 7-fold higher cellular uptake than the control photosensitizer tetraphenylporphyrin (TPP) after 24 hours incubation with KB nasopharyngeal cells, overexpressing folic acid receptors. Folic acid competitively inhibited tumor cellular uptake by up to 70%, suggesting active transport across the cell membrane via folate receptor-mediated endocytosis. The conjugates also showed photocytotoxicity toward KB cells, whereas the control photosensitizer TPP was not photoactive. These results indicate the feasibility of using folic acid as a targeting molecule to guide photosensitizing agents to target cells.

Folic acid can also be used to modify drug delivery systems to achieve high-payload drug delivery. A pH-sensitive and folic acid receptor-targeted liposome designed to deliver water-soluble photosensitizer chloroaluminum phthalocyanine tetrasulfonate ([AIPcS₍₄₎]⁽⁴⁻⁾) to tumor cells was prepared.¹¹⁵ This dual-targeting formulation was significantly more phototoxic than free [AIPcS₍₄₎]⁽⁴⁻⁾ and liposomal formulations that were either pH-sensitive or folic acid receptor-targeted only. A novel approach to targeting folic acid receptors using LDL particles was recently reported.¹¹⁶ As described above, the same group reconstituted endogenous LDL to photodynamically target LDL receptors.^{105,106} Because ApoB-100 protein on LDL is responsible for LDL-receptor recognition and binding, it is hypothesized that modifying LDL ApoB-100 will abolish its LDL receptor-binding capacity and, more importantly, be able to reroute the modified LDL to a new target. As a proof of concept, a LDL-based folic acid receptor-targeted nanoparticle was prepared by conjugating folic acid to the Lys residues of ApoB-100 protein, and the photosensitizer tetrabutyl-silicon phthalocyanine bisoleate was reconstituted into the LDL lipid core. This novel LDL nanoparticle had a high photosensitizer payload (molar ratio: 300 to 1) and was demonstrated using confocal microscopy and flow

cytometry studies to have significant cellular photosensitizer uptake in folic acid receptor-overexpressing KB cells. Competitive uptake inhibition by free folic acid, combined with a lack of photosensitizer accumulation in folic acid receptor-negative cells (CHO and HT-1080 cell lines) and LDL receptor-overexpressing HepG₂ cells, indicates that folic acid conjugation to the Lys side-chain amino groups of ApoB-100 blocks binding to the LDL receptor and reroutes the resulting conjugate to cancer cells via the folic acid pathway.

6. Glucose Transporters

Glucose transporters (GLUTs) are cell membrane proteins responsible for the uptake of glucose by all types of cells. Cancer cells generally overexpress GLUTs due to their increased energy requirements, and the overexpression of GLUTs are associated with tumor metastasis and poor prognosis.¹¹⁷ GLUT overexpression in human cancers has been explored in tumor positron emission tomography (PET) using 2-fluoro-2-deoxyglucose (¹⁸FDG), which is widely used in tumor detection. On the basis of the same principle, a near-infrared fluorescence imaging and photosensitizing agent targeting tumor GLUTs was prepared by conjugating photosensitizer pyropheophorbide with 2-deoxyglucose.¹¹⁸ The resultant pyropheophorbide 2-deoxyglucosamide (Pyro-2DG) was able to accumulate in tumor cells through GLUTs. Fluorescence-imaging studies demonstrated that Pyro-2DG was selectively retained in the 9L glioma rat tumor. Photoactivation of Pyro-2DG induced selective mitochondrial damage in the tumor tissue. The development of probes with both tumor imaging and targeting functions makes it possible to use a single agent to treat the tumor tissue under the guidance of tumor imaging.

A thio derivative of glucose was conjugated with photosensitizer tetra(pentafluorophenyl) porphyrin, and the resulting conjugate was shown to be actively uptaken by tumor cells via GLUTs and exhibited enhanced photocytotoxicity.¹¹⁹ Interestingly, glucose conjugation with meta-hydroxyphenyl porphyrin (m-THPP) and meta-hydroxyphenyl chlorin (m-THPC) did not signi-

ficantly reduce the singlet oxygen yields compared to nonglucosylated photosensitizers,¹²⁰ indicating that glucose conjugation of existing photosensitizers could be an effective way to improve tumor cell targeting.

7. Integrin Receptors

Integrins are heterodimeric transmembrane cell-adhesion proteins that promote the attachment and migration of cells to the surrounding extracellular matrix (ECM).¹²¹ They are composed of noncovalently bound α - and β -subunits, the N-terminal domains of which are combined to form a ligand-binding site. Several integrins (e.g., $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$) play important roles in regulating tumor growth, angiogenesis, and metastasis and have been recognized as promising anticancer targets. A phthalocyanine photosensitizer AlPcS₄ was covalently conjugated to adenovirus type 2 capsid proteins containing the arginine-glycine-aspartic acid (RGD) motif that is known to have high specificity and affinity to the $\alpha_v\beta_3$ integrin.¹²² To minimize the adverse influence of chemical conjugation on the biological activities of the photosensitizer and protein, one or two caproic acid spacer chains were inserted between the photosensitizer and the adenovirus protein. Despite the effort, AlPcS₄ and virus protein conjugates were still much lower than the free photosensitizer in singlet oxygen production. Nevertheless, the conjugate with two caproic acid spacer chains was more phototoxic than the conjugate with one caproic acid spacer chain and the free AlPcS₄ in human A549 and Hep2 cell lines.

8. Insulin Receptors

The photodynamic targeting of tumor cells can be achieved by targeting the insulin receptor, which is an internalizing cell membrane receptor. Chlorin e6 was covalently coupled to insulin via a BSA carrier.^{123–125} The resultant chlorin e6-BSA-insulin conjugate had high-binding affinity for insulin receptors and could be internalized via an active receptor-mediated endocytosis pathway in human hepatoma cell line PLC/PRF/5. Fluores-

cence labeling the conjugate with FITC demonstrated that the conjugate was localized around and within the cell nucleus following endocytosis. The phototoxicity of insulin receptor-targeted sensitizer conjugate was up to 100 times higher than the free chlorin e6, which could be competitively inhibited by receptor ligands.

9. Nuclear Targeting

Because most photosensitizers have low accumulation levels in the cell nucleus, the nucleus is generally not considered as a major target for PDT using common photosensitizers, even though the nucleus is highly sensitive to ROS damage.¹²⁶ To obtain nuclear delivery of photosensitizers, Sobolev and colleagues linked variants of the simian virus SV40 large tumor antigen nuclear localization signal (NLS) to the previously mentioned chlorin e6-BSA-insulin conjugate, either by peptide cross-linking to BSA or by encoding the sequence within that of a β -galactosidase fusion-protein carrier.^{127,128} The insulin moiety on the conjugate allowed the internalization of conjugate after binding to insulin receptors. Subsequently, the NLS components directed the conjugate to the nucleus. The NLS-chlorin e6-BSA-insulin conjugate dramatically increased the phototoxicity of chlorin e6 in PLC/PRF/5 human hepatoma cells. Chlorin e6 conjugated with β -galactosidase-NLS fusion protein (P10) produced the most phototoxic conjugate, being 2400-fold more cytotoxic than free chlorin e6 and 15-fold more cytotoxic than a NLS-deficient β -galactosidase-(chlorin e6)-insulin construct. This result demonstrates the extraordinary potency of nuclear targeting.

A bacteria-expressed recombinant transporter that can deliver a photosensitizer to a cancer cell nucleus has been reported.¹²⁹ It is comprised of four components: α -melanocyte-stimulating hormone (MSH) as an internalizing cell-specific ligand, the NLS from the SV40 large tumor antigen for nuclear drug delivery, an *E. coli* hemoglobin-like protein (HMP) as a carrier, and an endosomolytic polypeptide from diphtheria toxin (DTox) for endosome disruption following internalization. This DTox-HMP-NLS-MSH construct was shown to specifically deliver photosen-

sitizer bacteriochlorin p6 to the nuclei of MSH receptor-positive M3 mouse melanoma cells, but not MSH receptor-negative mouse fibroblast cells. Consequently, the photocytotoxicity of bacteriochlorin p6-DT_{ox}-HMP-NLS-MSH was about 230 times higher than that of free bacteriochlorin p6 (IC₅₀: 22 nm versus 4990 nm) in the MSH receptor-overexpressing mouse B16-F1 melanoma cells, whereas it was not phototoxic at all in MSH receptor-negative cells. Interestingly, the construct lacking an endosomolytic moiety DT_{ox} or NLS was about 5 and 35 times less effective, respectively, than the complete construct, confirming the importance of nuclear targeting by PDT.

Since steroid hormone receptors are nuclear receptors, conjugating photosensitizers with steroid hormones provides another possibility of targeting the cell nucleus. A conjugate of a C(11)-beta-derivative of estradiol and an asymmetric tetraphenylporphyrin has been synthesized to target estrogen receptor-overexpressing breast tumor cells.^{130,131} A radioligand-binding assay indicated that this conjugate was able to bind to estrogen receptors in a dose-dependent manner, even though the binding affinity was about 274 times less than the natural ligand estradiol. Cellular uptake of photosensitizer-estradiol conjugate was significantly higher in the estrogen receptor-positive MCF-7 human breast cancer cells than the receptor-negative HS578t cells and could be competitively inhibited by co-incubation with estradiol. These results indicate that photosensitizer steroid hormone conjugates can be used to target hormone-sensitive cancers, such as breast, ovarian, and prostate cancers.

V. CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Clearly, PDT is being actively pursued for targeting either tumor vasculature or cells. Numerous new photosensitizer conjugates have been constructed to target various cellular markers on tumor or endothelial cells. However, few of these new constructs are close to the clinical trial stage. For many photosensitizer conjugates, drug development is unfortunately limited to the stage of in vitro testing. A number of factors related to photosensitizing agents, targeting molecules, and

tumor tissues are responsible for this formidable fact. First, for most hydrophobic photosensitizers, the strong tendency of aggregation makes chemical conjugation and purification difficult. Factors like aggregation of not only photosensitizers but also photosensitizer-targeting molecules and a lack of conjugate stability could certainly compromise drug analysis, pharmacokinetic properties, and biological efficacy. Second, chemical manipulations on targeting molecules (e.g., antibodies, ligands) required for conjugation procedures often result in decreased affinity to their intended tumor marker targets and increased nonspecific interactions, as shown in many previous studies. Third, all the tumor or endothelial cell markers discovered so far are not exclusively expressed on the target cells. They are also expressed on normal cells, although to a lower extent. Moreover, the expression of tumor and endothelial cell markers are neither stable nor homogeneous. These factors will inevitably affect the specificity and efficacy of targeted PDT. Finally, tumor tissues tend to generate various physiological barriers (e.g., heterogeneity in vascular permeability and perfusion, high interstitial pressure), which will further decrease the effectiveness of targeted therapy by limiting the uptake and penetration of targeting molecules.

Being relatively simple in principle and photosensitizer production, passive vascular-targeting PDT using nonconjugated photosensitizers is effective in avoiding some major problems associated with the active targeting PDT mentioned above, and this approach has demonstrated a superior therapeutic advantage in both preclinical and clinical studies. As a result, passive vascular-targeting PDT has been accepted in medical practice, and more regulatory approvals are expected in the near future. The vascular-targeting approach offers several unique advantages in target accessibility and targeting efficiency over the tumor cellular-targeting strategy. With the advent of more and more tumor vascular markers, the active photodynamic vascular-targeting approach, which is currently emerging, will certainly bear fruit in the future.

It may be the case that vascular targeting alone will not be enough to completely eradicate tumor tissue.¹⁵ The combination of tumor vascular and cellular targeting is likely to be a more

practical way of achieving tumor control. Several approaches, such as the combined PDT using both vascular-targeting and cellular-targeting photosensitizers,¹³² photosensitizer dose fractionation in vascular and cellular compartments,¹³³ and temporal PDT targeting of tumor cells and vasculature²² have all been proposed to target both tumor blood vessels and tumor cells for an enhanced therapeutic outcome. With the development of more targeted photosensitizer agents, the possible synergism from the combination of active PDT targeting of tumor vasculature and tumor cells should be explored.

Finally, recent advances in nanotechnology provide a tremendous momentum to construct multifunctional nanophotomedicine platforms.¹³⁴ Photosensitizers can be incorporated into various polymeric nanoparticles or nanodevices modified with both tumor imaging and targeting moieties. The resultant nanophotomedicines could serve as both a tumor diagnostic and targeted therapeutic agent. More importantly, these nano drug delivery systems can be specially fabricated to have multiple targeting capabilities and controlled drug release triggered by tumor pathological environment factors (e.g., pH, enzyme activities), magnetic field, and light. The construction of such multifunction and multitargeting nanophotomedicines may allow tumor imaging, targeted tumor therapy, and therapeutic response monitoring in one single entity.

ACKNOWLEDGMENTS

This work was supported by Department of Defense (DOD) Grant W81XWH-06-1-0148 and National Cancer Institute Grant PO1CA84203.

REFERENCES

1. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbek M, Moan J, Peng Q. Photodynamic therapy. *J Natl Cancer Inst.* 1998;90:889–905.
2. Schmidt R. Photosensitized generation of singlet oxygen. *Photochem Photobiol.* 2006;82:1161–77.
3. Moan J, Wold E. Detection of singlet oxygen production by ESR. *Nature.* 1979;279:450–1.
4. Niedre MJ, Yu CS, Patterson MS, Wilson BC. Singlet oxygen luminescence as an in vivo photodynamic

- therapy dose metric: validation in normal mouse skin with topical amino-levulinic acid. *Br J Cancer*. 2005;92:298–304.
5. Detty MR, Gibson SL, Wagner SJ. Current clinical and preclinical photosensitizers for use in photodynamic therapy. *J Med Chem*. 2004;47:3897–915.
 6. Konan YN, Gurny R, Allemann E. State of the art in the delivery of photosensitizers for photodynamic therapy. *J Photochem Photobiol B*. 2002;66:89–106.
 7. Hsi RA, Rosenthal DI, Glatstein E. Photodynamic therapy in the treatment of cancer: current state of the art. *Drugs*. 1999;57:725–34.
 8. Boyle RW, Dolphin D. Structure and biodistribution relationships of photodynamic sensitizers. *Photochem Photobiol*. 1996;64:469–85.
 9. Nadeau V, O'Dwyer M, Hamdan K, Tait I, Padgett M. In vivo measurement of 5-aminolaevulinic acid-induced protoporphyrin IX photobleaching: a comparison of red and blue light of various intensities. *Photodermatol Photoimmunol Photomed*. 2004;20:170–4.
 10. Henderson BW, Busch TM, Snyder JW. Fluence rate as a modulator of PDT mechanisms. *Lasers Surg Med*. 2006;38:489–93.
 11. Henderson BW, Dougherty TJ. How does photodynamic therapy work? *Photochem Photobiol*. 1992;55:145–57.
 12. Foster TH, Hartley DF, Nichols MG, Hilf R. Fluence rate effects in photodynamic therapy of multicell tumor spheroids. *Cancer Res*. 1993;53:1249–54.
 13. Foster TH, Murant RS, Bryant RG, Knox RS, Gibson SL, Hilf R. Oxygen consumption and diffusion effects in photodynamic therapy. *Radiat Res*. 1991;126:296–303.
 14. Siemann DW, Bibby MC, Dark GG, Dicker AP, Eskens FA, Horsman MR, Marme D, Lorusso PM. Differentiation and definition of vascular-targeted therapies. *Clin Cancer Res*. 2005;11:416–20.
 15. Siemann DW, Chaplin DJ, Horsman MR. Vascular-targeting therapies for treatment of malignant disease. *Cancer*. 2004;100:2491–9.
 16. Sharman WM, van Lier JE, Allen CM. Targeted photodynamic therapy via receptor mediated delivery systems. *Adv Drug Deliv Rev*. 2004;56:53–76.
 17. Schmidt-Erfurth U, Hasan T. Mechanisms of action of photodynamic therapy with verteporfin for the treatment of age-related macular degeneration. *Surv Ophthalmol*. 2000;45:195–214.
 18. Richter AM, Waterfield E, Jain AK, Canaan AJ, Allison BA, Levy JG. Liposomal delivery of a photosensitizer, benzoporphyrin derivative monoacid ring A (BPD), to tumor tissue in a mouse tumor model. *Photochem Photobiol*. 1993;57:1000–6.
 19. Allison BA, Pritchard PH, Levy JG. Evidence for low-density lipoprotein receptor-mediated uptake of benzoporphyrin derivative. *Br J Cancer*. 1994;69:833–9.
 20. Kramer M, Miller JW, Michaud N, Moulton RS, Hasan T, Flotte TJ, Gragoudas ES. Liposomal benzoporphyrin derivative verteporfin photodynamic therapy. Selective treatment of choroidal neovascularization in monkeys. *Ophthalmology*. 1996;103:427–38.
 21. Chen B, Pogue BW, Goodwin IA, O'Hara JA, Wilmot CM, Hutchins JE, Hoopes PJ, Hasan T. Blood flow dynamics after photodynamic therapy with verteporfin in the RIF-1 tumor. *Radiat Res*. 2003;160:452–9.
 22. Chen B, Pogue BW, Hoopes PJ, Hasan T. Combining vascular and cellular targeting regimens enhances the efficacy of photodynamic therapy. *Int J Radiat Oncol Biol Phys*. 2005;61:1216–26.
 23. Finger VH, Kik PK, Haydon PS, Cerrito PB, Tseng M, Abang E, Wieman TJ. Analysis of acute vascular damage after photodynamic therapy using benzoporphyrin derivative (BPD). *Br J Cancer*. 1999;79:1702–8.
 24. Kurohane K, Tominaga A, Sato K, North JR, Namba Y, Oku N. Photodynamic therapy targeted to tumor-induced angiogenic vessels. *Cancer Lett*. 2001;167:49–56.
 25. Chen B, Pogue BW, Luna JM, Hardman RL, Hoopes PJ, Hasan T. Tumor vascular permeabilization by vascular-targeting photosensitization: effects, mechanism, and therapeutic implications. *Clin Cancer Res*. 2006;12:917–23.
 26. Cramers P, Ruevekamp M, Oppelaar H, Dalesio O, Baas P, Stewart FA. Foscan uptake and tissue distribution in relation to photodynamic efficacy. *Br J Cancer*. 2003;88:283–90.
 27. Triesscheijn M, Ruevekamp M, Aalders M, Baas P, Stewart FA. Outcome of mTHPC mediated photodynamic therapy is primarily determined by the vascular response. *Photochem Photobiol*. 2005;81:1161–7.
 28. Veenhuizen RB, Oppelaar H, Ruevekamp M, Schellens J, Dalesio O, Stewart FA. Does tumor uptake of Foscan determine PDT efficacy? *Int J Cancer*. 1997;73:236–9.
 29. Veenhuizen RB, Ruevekamp MC, Oppelaar H, Helmerhorst TJ, Kenemans P, Stewart FA. Foscan-mediated photodynamic therapy for a peritoneal-cancer model: drug distribution and efficacy studies. *Int J Cancer*. 1997;73:230–5.
 30. Weersink RA, Forbes J, Bisland S, Trachtenberg J, Elhilali M, Brun PH, Wilson BC. Assessment of cutaneous photosensitivity of TOOKAD (WST09) in preclinical animal models and in patients. *Photochem Photobiol*. 2005;81:106–13.
 31. Chen Q, Huang Z, Luck D, Beckers J, Brun PH, Wilson BC, Scherz A, Salomon Y, Hetzel FW. Pre-clinical studies in normal canine prostate of a novel palladium-bacteriopheophorbide (WST09) photosensitizer for photodynamic therapy of prostate cancers. *Photochem Photobiol*. 2002;76:438–45.
 32. Weersink RA, Bogaards A, Gertner M, Davidson SR, Zhang K, Netchev G, Trachtenberg J, Wilson BC. Techniques for delivery and monitoring of TOOKAD

- (WST09)-mediated photodynamic therapy of the prostate: clinical experience and practicalities. *J Photochem Photobiol B*. 2005;79:211–22.
33. Woodhams JH, MacRobert AJ, Novelli M, Bown SG. Photodynamic therapy with WST09 (Tookad): quantitative studies in normal colon and transplanted tumours. *Int J Cancer*. 2006;118:477–82.
 34. Koudinova NV, Pinthus JH, Brandis A, Brenner O, Bendel P, Ramon J, Eshhar Z, Scherz A, Salomon Y. Photodynamic therapy with Pd-Bacteriopheophorbide (TOOKAD): successful in vivo treatment of human prostatic small cell carcinoma xenografts. *Int J Cancer*. 2003;104:782–9.
 35. Reinke MH, Canakis C, Husain D, Michaud N, Flotte TJ, Gragoudas ES, Miller JW. Verteporfin photodynamic therapy retreatment of normal retina and choroid in the cynomolgus monkey. *Ophthalmology*. 1999;106:1915–23.
 36. Miller JW, Schmidt-Erfurth U, Sickenberg M, Pournaras CJ, Laqua H, Barbazetto I, Zografos L, Piguet B, Donati G, Lane AM, Birngruber R, van den Berg H, Strong A, Manjuri U, Gray T, Fsadni M, Bressler NM, Gragoudas ES. Photodynamic therapy with verteporfin for choroidal neovascularization caused by age-related macular degeneration: results of a single treatment in a phase 1 and 2 study. *Arch Ophthalmol*. 1999;117:1161–73.
 37. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science*. 2005;307:58–62.
 38. Borle F, Radu A, Fontolliet C, van den Bergh H, Monnier P, Wagnieres G. Selectivity of the photosensitizer Tookad for photodynamic therapy evaluated in the Syrian golden hamster cheek pouch tumour model. *Br J Cancer*. 2003;89:2320–6.
 39. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev*. 2005;15:102–11.
 40. McDonald DM, Choyke PL. Imaging of angiogenesis: from microscope to clinic. *Nat Med*. 2003;9:713–25.
 41. Szala S. Two-domain vascular disruptive agents in cancer therapy. *Curr Cancer Drug Targets*. 2004;4:501–9.
 42. Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat Rev Cancer*. 2003;3:422–33.
 43. Borsi L, Balza E, Carnemolla B, Sassi F, Castellani P, Berndt A, Kosmehl H, Biro A, Siri A, Orecchia P, Grassi J, Neri D, Zardi L. Selective targeted delivery of TNF α to tumor blood vessels. *Blood*. 2003;102:4384–92.
 44. Santimaria M, Moscatelli G, Viale GL, Giovannoni L, Neri G, Viti F, Leprini A, Borsi L, Castellani P, Zardi L, Neri D, Riva P. Immunoscintigraphic detection of the ED-B domain of fibronectin, a marker of angiogenesis, in patients with cancer. *Clin Cancer Res*. 2003;9:571–9.
 45. Birchler M, Viti F, Zardi L, Spiess B, Neri D. Selective targeting and photocoagulation of ocular angiogenesis mediated by a phage-derived human antibody fragment. *Nat Biotechnol*. 1999;17:984–8.
 46. Renno RZ, Terada Y, Haddadin MJ, Michaud NA, Gragoudas ES, Miller JW. Selective photodynamic therapy by targeted verteporfin delivery to experimental choroidal neovascularization mediated by a homing peptide to vascular endothelial growth factor receptor-2. *Arch Ophthalmol*. 2004;122:1002–11.
 47. Perret GY, Starzec A, Hauet N, Vergote J, Le Pecqueur M, Vassy R, Leger G, Verbeke KA, Bormans G, Nicolas P, Verbruggen AM, Moretti JL. In vitro evaluation and biodistribution of a ^{99m}Tc -labeled anti-VEGF peptide targeting neuropilin-1. *Nucl Med Biol*. 2004;31:575–81.
 48. Chen C, Li M, Chai H, Yang H, Fisher WE, Yao Q. Roles of neuropilins in neuronal development, angiogenesis, and cancers. *World J Surg*. 2005;29:271–5.
 49. Tirand L, Frochot C, Vanderesse R, Thomas N, Trinquet E, Pinel S, Viriot ML, Guillemin F, Barberi-Heyob M. A peptide competing with VEGF165 binding on neuropilin-1 mediates targeting of a chlorin-type photosensitizer and potentiates its photodynamic activity in human endothelial cells. *J Control Release*. 2006;111:153–64.
 50. Ichikawa K, Hikita T, Maeda N, Yonezawa S, Takeuchi Y, Asai T, Namba Y, Oku N. Antiangiogenic photodynamic therapy (PDT) by using long-circulating liposomes modified with peptide specific to angiogenic vessels. *Biochim Biophys Acta*. 2005;1669:69–74.
 51. Fried NM. Therapeutic applications of lasers in urology: an update. *Expert Rev Med Devices*. 2006;3:81–94.
 52. Panjehpour M, Overholt BF, Haydek JM. Light sources and delivery devices for photodynamic therapy in the gastrointestinal tract. *Gastrointest Endosc Clin N Am*. 2000;10:513–32.
 53. Haylett AK, Higley K, Chiu M, Shackley DC, Moore JV. Collagen secretion after photodynamic therapy versus scar-inducing anti-cancer modalities: an in vitro study. *Photochem Photobiol Sci*. 2002;1:673–7.
 54. Chang SC, Buonaccorsi G, MacRobert A, Bown SG. Interstitial and transurethral photodynamic therapy of the canine prostate using meso-tetra-(m-hydroxyphenyl) chlorin. *Int J Cancer*. 1996;67:555–62.
 55. Hopper C. Photodynamic therapy: a clinical reality in the treatment of cancer. *Lancet Oncol*. 2000;1:212–9.
 56. Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Adv Enzyme Regul*. 2001;41:189–207.
 57. Jori G, Reddi E. The role of lipoproteins in the delivery of tumour-targeting photosensitizers. *Int J Biochem*. 1993;25:1369–75.
 58. Allen CM, Langlois R, Sharman WM, La Madeleine C, Van Lier JE. Photodynamic properties of amphiphilic derivatives of aluminum tetrasulfophthalocyanine. *Photochem Photobiol*. 2002;76:208–16.
 59. Van de Putte M, Roskams T, Vandenheede JR,

- Agostinis P, de Witte PA. Elucidation of the tumorigenic principle of hypericin. *Br J Cancer*. 2005;92:1406–13.
60. Sheyhedin I, Aizawa K, Araake M, Kumasaka H, Okunaka T, Kato H. The effects of serum on cellular uptake and phototoxicity of mono-L-aspartyl chlorin e6 (NPe6) in vitro. *Photochem Photobiol*. 1998;68:110–4.
 61. Mori M, Kuroda T, Obana A, Sakata I, Hirano T, Nakajima S, Hikida M, Kumagai T. In vitro plasma protein binding and cellular uptake of ATX-S10(Na), a hydrophilic chlorin photosensitizer. *Jpn J Cancer Res*. 2000;91:845–52.
 62. Kessel D, Whitcomb KL, Schulz V. Lipoprotein-mediated distribution of N-aspartyl chlorin-E6 in the mouse. *Photochem Photobiol*. 1992;56:51–6.
 63. Almeida RD, Manadas BJ, Carvalho AP, Duarte CB. Intracellular signaling mechanisms in photodynamic therapy. *Biochim Biophys Acta*. 2004;1704:59–86.
 64. Piette J, Volanti C, Vantieghem A, Matroule JY, Habraken Y, Agostinis P. Cell death and growth arrest in response to photodynamic therapy with membrane-bound photosensitizers. *Biochem Pharmacol*. 2003;66:1651–9.
 65. Ferrario A, Fisher AM, Rucker N, Gomer CJ. Celecoxib and NS-398 enhance photodynamic therapy by increasing in vitro apoptosis and decreasing in vivo inflammatory and angiogenic factors. *Cancer Res*. 2005;65:9473–8.
 66. Mew D, Wat CK, Towers GH, Levy JG. Photoimmunotherapy: treatment of animal tumors with tumor-specific monoclonal antibody-hematoporphyrin conjugates. *J Immunol*. 1983;130:1473–7.
 67. Mew D, Lum V, Wat CK, Towers GH, Sun CH, Walter RJ, Wright W, Berns MW, Levy JG. Ability of specific monoclonal antibodies and conventional antisera conjugated to hematoporphyrin to label and kill selected cell lines subsequent to light activation. *Cancer Res*. 1985;45:4380–6.
 68. Pogrebniak HW, Matthews W, Black C, Russo A, Mitchell JB, Smith P, Roth JA, Pass HI. Targeted phototherapy with sensitizer-monoclonal antibody conjugate and light. *Surg Oncol*. 1993;2:31–42.
 69. Oseroff AR, Ohuoha D, Hasan T, Bommer JC, Yarmush ML. Antibody-targeted photolysis: selective photodestruction of human T-cell leukemia cells using monoclonal antibody-chlorin e6 conjugates. *Proc Natl Acad Sci U S A*. 1986;83:8744–8.
 70. Oseroff AR, Ara G, Ohuoha D, Aprille J, Bommer JC, Yarmush ML, Foley J, Cincotta L. Strategies for selective cancer photochemotherapy: antibody-targeted and selective carcinoma cell photolysis. *Photochem Photobiol*. 1987;46:83–96.
 71. Jiang FN, Jiang S, Liu D, Richter A, Levy JG. Development of technology for linking photosensitizers to a model monoclonal antibody. *J Immunol Methods*. 1990;134:139–49.
 72. Jiang FN, Liu DJ, Neyndorff H, Chester M, Jiang SY, Levy JG. Photodynamic killing of human squamous cell carcinoma cells using a monoclonal antibody-photosensitizer conjugate. *J Natl Cancer Inst*. 1991;83:1218–25.
 73. Jiang FN, Richter AM, Jain AK, Levy JG, Smits C. Biodistribution of a benzoporphyrin derivative-monoclonal antibody conjugate in A549-tumor-bearing nude mice. *Biotechnol Ther*. 1993;4:43–61.
 74. Goff BA, Bamberg M, Hasan T. Photoimmunotherapy of human ovarian carcinoma cells ex vivo. *Cancer Res*. 1991;51:4762–7.
 75. Goff BA, Hermanto U, Rumbaugh J, Blake J, Bamberg M, Hasan T. Photoimmunotherapy and biodistribution with an OC125-chlorin immunoconjugate in an in vivo murine ovarian cancer model. *Br J Cancer*. 1994;70:474–80.
 76. Goff BA, Blake J, Bamberg MP, Hasan T. Treatment of ovarian cancer with photodynamic therapy and immunoconjugates in a murine ovarian cancer model. *Br J Cancer*. 1996;74:1194–8.
 77. Hamblin MR, Miller JL, Hasan T. Effect of charge on the interaction of site-specific photoimmunconjugates with human ovarian cancer cells. *Cancer Res*. 1996;56:5205–10.
 78. Duska LR, Hamblin MR, Bamberg MP, Hasan T. Biodistribution of charged F(ab')₂ photoimmunconjugates in a xenograft model of ovarian cancer. *Br J Cancer*. 1997;75:837–44.
 79. Molpus KL, Hamblin MR, Rizvi I, Hasan T. Intraperitoneal photoimmunotherapy of ovarian carcinoma xenografts in nude mice using charged photoimmunconjugates. *Gynecol Oncol*. 2000;76:397–404.
 80. Del Governatore M, Hamblin MR, Piccinini EE, Ugolini G, Hasan T. Targeted photodestruction of human colon cancer cells using charged 17.1A chlorin e6 immunoconjugates. *Br J Cancer*. 2000;82:56–64.
 81. Hamblin MR, Del Governatore M, Rizvi I, Hasan T. Biodistribution of charged 17.1A photoimmunconjugates in a murine model of hepatic metastasis of colorectal cancer. *Br J Cancer*. 2000;83:1544–51.
 82. Del Governatore M, Hamblin MR, Shea CR, Rizvi I, Molpus KG, Tanabe KK, Hasan T. Experimental photoimmunotherapy of hepatic metastases of colorectal cancer with a 17.1A chlorin(e6) immunoconjugate. *Cancer Res*. 2000;60:4200–5.
 83. Rakestraw SL, Tompkins RG, Yarmush ML. Antibody-targeted photolysis: in vitro studies with Sn(IV) chlorin e6 covalently bound to monoclonal antibodies using a modified dextran carrier. *Proc Natl Acad Sci U S A*. 1990;87:4217–21.
 84. Rakestraw SL, Tompkins RG, Yarmush ML. Preparation and characterization of immunoconjugates for antibody-targeted photolysis. *Bioconjug Chem*. 1990;1:212–21.
 85. Rakestraw SL, Ford WE, Tompkins RG, Rodgers MA, Thorpe WP, Yarmush ML. Antibody-targeted photolysis: in vitro immunological, photophysical, and cytotoxic properties of monoclonal antibody-dextran-Sn(IV) chlorin e6 immunoconjugates. *Biotechnol Prog*. 1992;8:30–9.

86. Hu LK, Hasan T, Gragoudas ES, Young LH. Photoimmunotherapy of human uveal melanoma cells. *Exp Eye Res.* 1995;61:385–91.
87. Carcenac M, Larroque C, Langlois R, van Lier JE, Artus JC, Pelegrin A. Preparation, phototoxicity and biodistribution studies of anti-carcinoembryonic antigen monoclonal antibody-phthalocyanine conjugates. *Photochem Photobiol.* 1999;70:930–6.
88. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000;100:57–70.
89. Soukos NS, Hamblin MR, Keel S, Fabian RL, Deutsch TF, Hasan T. Epidermal growth factor receptor-targeted immunophotodiagnosis and photoimmunotherapy of oral precancer in vivo. *Cancer Res.* 2001;61:4490–6.
90. Gijssens A, De Witte P. Photocytotoxic action of EGF-PVA-Sn(IV)chlorin e6 and EGF-dextran-Sn(IV)chlorin e6 internalizable conjugates on A431 cells. *Int J Oncol.* 1998;13:1171–7.
91. Gijssens A, Missiaen L, Merlevede W, de Witte P. Epidermal growth factor-mediated targeting of chlorin e6 selectively potentiates its photodynamic activity. *Cancer Res.* 2000;60:2197–202.
92. Savellano MD, Hasan T. Targeting cells that overexpress the epidermal growth factor receptor with polyethylene glycolated BPD verteporfin photosensitizer immunoconjugates. *Photochem Photobiol.* 2003;77:431–9.
93. Savellano MD, Hasan T. Photochemical targeting of epidermal growth factor receptor: a mechanistic study. *Clin Cancer Res.* 2005;11:1658–68.
94. Hemming AW, Davis NL, Dubois B, Quenville NF, Finley RJ. Photodynamic therapy of squamous cell carcinoma. An evaluation of a new photosensitizing agent, benzoporphyrin derivative and new photoimmunconjugate. *Surg Oncol.* 1993;2:187–96.
95. Vrouenraets MB, Visser GW, Stewart FA, Stigter M, Oppelaar H, Postmus PE, Snow GB, van Dongen GA. Development of meta-tetrahydroxyphenylchlorin-monoclonal antibody conjugates for photoimmunotherapy. *Cancer Res.* 1999;59:1505–13.
96. Carcenac M, Dorvillius M, Garambois V, Glaussel F, Larroque C, Langlois R, Hynes NE, van Lier JE, Pelegrin A. Internalisation enhances photo-induced cytotoxicity of monoclonal antibody-phthalocyanine conjugates. *Br J Cancer.* 2001;85:1787–93.
97. Hudson R, Carcenac M, Smith K, Madden L, Clarke OJ, Pelegrin A, Greenman J, Boyle RW. The development and characterisation of porphyrin isothiocyanate-monoclonal antibody conjugates for photoimmunotherapy. *Br J Cancer.* 2005;92:1442–9.
98. Vrouenraets MB, Visser GW, Loup C, Meunier B, Stigter M, Oppelaar H, Stewart FA, Snow GB, van Dongen GA. Targeting of a hydrophilic photosensitizer by use of internalizing monoclonal antibodies: a new possibility for use in photodynamic therapy. *Int J Cancer.* 2000;88:108–14.
99. Vrouenraets MB, Visser GW, Stigter M, Oppelaar H, Snow GB, van Dongen GA. Targeting of aluminium (III) phthalocyanine tetrasulfonate by use of internalizing monoclonal antibodies: improved efficacy in photodynamic therapy. *Cancer Res.* 2001;61:1970–5.
100. Vrouenraets MB, Visser GW, Stigter M, Oppelaar H, Snow GB, van Dongen GA. Comparison of aluminium (III) phthalocyanine tetrasulfonate- and meta-tetrahydroxyphenylchlorin-monoclonal antibody conjugates for their efficacy in photodynamic therapy in vitro. *Int J Cancer.* 2002;98:793–8.
101. Savellano MD, Pogue BW, Hoopes PJ, Vitetta ES, Paulsen KD. Multiepitope HER2 targeting enhances photoimmunotherapy of HER2-overexpressing cancer cells with pyropheophorbide-a immunoconjugates. *Cancer Res.* 2005;65:6371–9.
102. Chung NS, Wasan KM. Potential role of the low-density lipoprotein receptor family as mediators of cellular drug uptake. *Adv Drug Deliv Rev.* 2004;56:1315–34.
103. Hamblin MR, Newman EL. Photosensitizer targeting in photodynamic therapy. II. Conjugates of haematoporphyrin with serum lipoproteins. *J Photochem Photobiol B.* 1994;26:147–57.
104. Schmidt-Erfurth U, Diddens H, Birngruber R, Hasan T. Photodynamic targeting of human retinoblastoma cells using covalent low-density lipoprotein conjugates. *Br J Cancer.* 1997;75:54–61.
105. Zheng G, Li H, Zhang M, Lund-Katz S, Chance B, Glickson JD. Low-density lipoprotein reconstituted by pyropheophorbide cholesteryl oleate as target-specific photosensitizer. *Bioconjug Chem.* 2002;13:392–6.
106. Li H, Marotta DE, Kim S, Busch TM, Wileyto EP, Zheng G. High payload delivery of optical imaging and photodynamic therapy agents to tumors using phthalocyanine-reconstituted low-density lipoprotein nanoparticles. *J Biomed Opt.* 2005;10:41203.
107. Singh M. Transferrin as a targeting ligand for liposomes and anticancer drugs. *Curr Pharm Des.* 1999;5:443–51.
108. Hamblin MR, Newman EL. Photosensitizer targeting in photodynamic therapy. I. Conjugates of haematoporphyrin with albumin and transferrin. *J Photochem Photobiol B.* 1994;26:45–56.
109. Cavanaugh PG. Synthesis of chlorin e6-transferrin and demonstration of its light-dependent in vitro breast cancer cell killing ability. *Breast Cancer Res Treat.* 2002;72:117–30.
110. Gijssens A, Derycke A, Missiaen L, De Vos D, Huwyler J, Eberle A, de Witte P. Targeting of the photocytotoxic compound AIPcS4 to HeLa cells by transferrin conjugated PEG-liposomes. *Int J Cancer.* 2002;101:78–85.
111. Derycke AS, Kamuhabwa A, Gijssens A, Roskams T, De Vos D, Kasran A, Huwyler J, Missiaen L, de Witte PA. Transferrin-conjugated liposome targeting of photosensitizer AIPcS4 to rat bladder carcinoma cells. *J Natl Cancer Inst.* 2004;96:1620–30.
112. Derycke AS, De Witte PA. Transferrin-mediated

- targeting of hypericin embedded in sterically stabilized PEG-liposomes. *Int J Oncol.* 2002;20:181–7.
113. Lu Y, Low PS. Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv Drug Deliv Rev.* 2002;54:675–93.
114. Schneider R, Schmitt F, Frochot C, Fort Y, Lourette N, Guillemin F, Muller JF, Barberi-Heyob M. Design, synthesis, and biological evaluation of folic acid targeted tetraphenylporphyrin as novel photosensitizers for selective photodynamic therapy. *Bioorg Med Chem.* 2005;13:2799–808.
115. Qualls MM, Thompson DH. Chloroaluminum phthalocyanine tetrasulfonate delivered via acid-labile dipalmitoylcholine-folate liposomes: intracellular localization and synergistic phototoxicity. *Int J Cancer.* 2001;93:384–92.
116. Zheng G, Chen J, Li H, Glickson JD. Rerouting lipoprotein nanoparticles to selected alternate receptors for the targeted delivery of cancer diagnostic and therapeutic agents. *Proc Natl Acad Sci U S A.* 2005;102:17757–62.
117. Medina RA, Owen GI. Glucose transporters: expression, regulation and cancer. *Biol Res.* 2002;35:9–26.
118. Zhang M, Zhang Z, Blessington D, Li H, Busch TM, Madrak V, Miles J, Chance B, Glickson JD, Zheng G. Pyropheophorbide 2-deoxyglucosamide: a new photosensitizer targeting glucose transporters. *Bioconjug Chem.* 2003;14:709–14.
119. Chen X, Hui L, Foster DA, Drain CM. Efficient synthesis and photodynamic activity of porphyrin-saccharide conjugates: targeting and incapacitating cancer cells. *Biochemistry.* 2004;43:10918–29.
120. Bautista-Sanchez A, Kasselouri A, Desroches MC, Blais J, Maillard P, de Oliveira DM, Tedesco AC, Prognon P, Delaire J. Photophysical properties of glucoconjugated chlorins and porphyrins and their associations with cyclodextrins. *J Photochem Photobiol B.* 2005;81:154–62.
121. Jin H, Varner J. Integrins: roles in cancer development and as treatment targets. *Br J Cancer.* 2004;90:561–5.
122. Allen CM, Sharman WM, La Madeleine C, Weber JM, Langlois R, Ouellet R, van Lier JE. Photodynamic therapy: tumor targeting with adenoviral proteins. *Photochem Photobiol.* 1999;70:512–23.
123. Sobolev AS, Akhlynina TV, Yachmenev SV, Rosenkranz AA, Severin ES. Internalizable insulin-BSA-chlorin E6 conjugate is a more effective photosensitizer than chlorin E6 alone. *Biochem Int.* 1992;26:445–50.
124. Akhlynina TV, Rosenkranz AA, Jans DA, Gulak PV, Serebryakova NV, Sobolev AS. The use of internalizable derivatives of chlorin E6 for increasing its photosensitizing activity. *Photochem Photobiol.* 1993;58:45–8.
125. Akhlynina TV, Rosenkranz AA, Jans DA, Sobolev AS. Insulin-mediated intracellular targeting enhances the photodynamic activity of chlorin e6. *Cancer Res.* 1995;55:1014–9.
126. Rosenkranz AA, Jans DA, Sobolev AS. Targeted intracellular delivery of photosensitizers to enhance photodynamic efficiency. *Immunol Cell Biol.* 2000;78:452–64.
127. Akhlynina TV, Jans DA, Rosenkranz AA, Statsyuk NV, Balashova IY, Toth G, Pavo I, Rubin AB, Sobolev AS. Nuclear targeting of chlorin e6 enhances its photosensitizing activity. *J Biol Chem.* 1997;272:20328–31.
128. Akhlynina TV, Jans DA, Statsyuk NV, Balashova IY, Toth G, Pavo I, Rosenkranz AA, Naroditsky BS, Sobolev AS. Adenoviruses synergize with nuclear localization signals to enhance nuclear delivery and photodynamic action of internalizable conjugates containing chlorin e6. *Int J Cancer.* 1999;81:734–40.
129. Rosenkranz AA, Lunin VG, Gulak PV, Sergienko OV, Shumiantseva MA, Voronina OL, Gilyazova DG, John AP, Kofner AA, Mironov AF, Jans DA, Sobolev AS. Recombinant modular transporters for cell-specific nuclear delivery of locally acting drugs enhance photosensitizer activity. *FASEB J.* 2003;17:1121–3.
130. James DA, Swamy N, Paz N, Hanson RN, Ray R. Synthesis and estrogen receptor binding affinity of a porphyrin-estradiol conjugate for targeted photodynamic therapy of cancer. *Bioorg Med Chem Lett.* 1999;9:2379–84.
131. Swamy N, James DA, Mohr SC, Hanson RN, Ray R. An estradiol-porphyrin conjugate selectively localizes into estrogen receptor-positive breast cancer cells. *Bioorg Med Chem.* 2002;10:3237–43.
132. Peng Q, Warloe T, Moan J, Godal A, Apricena F, Giercksky KE, Nesland JM. Antitumor effect of 5-aminolevulinic acid-mediated photodynamic therapy can be enhanced by the use of a low dose of photofrin in human tumor xenografts. *Cancer Res.* 2001;61:5824–32.
133. Dolmans DE, Kadambi A, Hill JS, Flores KR, Gerber JN, Walker JP, Borel Rinkes IH, Jain RK, Fukumura D. Targeting tumor vasculature and cancer cells in orthotopic breast tumor by fractionated photosensitizer dosing photodynamic therapy. *Cancer Res.* 2002;62:4289–94.
134. Moghimi SM, Hunter AC, Murray JC. Nanomedicine: current status and future prospects. *FASEB J.* 2005;19:311–30.
135. Moshfeghi DM, Peyman GA, Moshfeghi AA, Khoobehi B, Primbs GB, Crean DH. Ocular vascular thrombosis following tin ethyl etiopurpurin (SnET2) photodynamic therapy: time dependencies. *Ophthalmic Surg Lasers.* 1998;29:663–8.
136. Primbs GB, Casey R, Wamser K, Snyder WJ, Crean DH. Photodynamic therapy for corneal neovascularization. *Ophthalmic Surg Lasers.* 1998;29:832–8.
137. Peyman GA, Moshfeghi DM, Moshfeghi A, Khoobehi B, Doiron DR, Primbs GB, Crean DH. Photodynamic therapy for choriocapillaris using tin ethyl etiopurpurin (SnET2). *Ophthalmic Surg Lasers.* 1997;28:409–17.
138. Criswell MH, Ciulla TA, Lowseth LA, Small W, Danis RP, Carson DL. Anastomotic vessels remain

- viable after photodynamic therapy in primate models of choroidal neovascularization. *Invest Ophthalmol Vis Sci.* 2005;46:2168–74.
139. Woodburn KW, Fan Q, Miles DR, Kessel D, Luo Y, Young SW. Localization and efficacy analysis of the phototherapeutic lutetium texaphyrin (PCI-0123) in the murine EMT6 sarcoma model. *Photochem Photobiol.* 1997;65:410–5.
 140. Blumenkranz MS, Woodburn KW, Qing F, Verdooner S, Kessel D, Miller R. Lutetium texaphyrin (Lu-TeX): a potential new agent for ocular fundus angiography and photodynamic therapy. *Am J Ophthalmol.* 2000;129:353–62.
 141. Miles D, Mody TD, Hatcher LI, Fiene J, Stiles M, Lin PP, Lee JW. Quantitation of motexafin lutetium in human plasma by liquid chromatography-tandem mass spectrometry and inductively coupled plasma-atomic emission spectroscopy. *AAPS PharmSci.* 2003;5:E23.
 142. Gomer CJ, Ferrario A. Tissue distribution and photosensitizing properties of mono-L-aspartyl chlorin e6 in a mouse tumor model. *Cancer Res.* 1990;50:3985–90.
 143. Saito K, Mikuniya N, Aizawa K. Effects of photodynamic therapy using mono-L-aspartyl chlorin e6 on vessels and its contribution to the antitumor effect. *Jpn J Cancer Res.* 2000;91:560–5.
 144. Nakashizuka T, Mori K, Hayashi N, Anzail K, Kanai K, Yoneya S, Moshfeghi DM, Peyman GA. Retreatment effect of NPe6 photodynamic therapy on the normal primate macula. *Retina.* 2001;21:493–8.
 145. Peyman GA, Kazi AA, Moshfeghi D, Unal M, Khoobehi B, Yoneya S, Mori K, Rivera I. Threshold and retreatment parameters of NPe6 photodynamic therapy in retinal and choroidal vessels. *Ophthalmic Surg Lasers.* 2000;31:323–7.
 146. Mori K, Yoneya S, Anzail K, Kabasawa S, Sodeyama T, Peyman GA, Moshfeghi DM. Photodynamic therapy of experimental choroidal neovascularization with a hydrophilic photosensitizer: mono-L-aspartyl chlorin e6. *Retina.* 2001;21:499–508.
 147. Chan AL, Juarez M, Allen R, Volz W, Albertson T. Pharmacokinetics and clinical effects of mono-L-aspartyl chlorin e6 (NPe6) photodynamic therapy in adult patients with primary or secondary cancer of the skin and mucosal surfaces. *Photodermatol Photoimmunol Photomed.* 2005;21:72–8.
 148. Harada M, Woodhams J, MacRobert AJ, Feneley MR, Kato H, Bown SG. The vascular response to photodynamic therapy with ATX-S10Na(II) in the normal rat colon. *J Photochem Photobiol B.* 2005;79:223–30.
 149. Obana A, Gohto Y, Kaneda K, Nakajima S, Takemura T, Miki T. Selective occlusion of choroidal neovascularization by photodynamic therapy with a water-soluble photosensitizer, ATX-S10. *Lasers Surg Med.* 1999;24:209–22.
 150. Gohto Y, Obana A, Kanai M, Nagata S, Nakajima S, Miki T. Treatment parameters for selective occlusion of experimental corneal neovascularization by photodynamic therapy using a water soluble photosensitizer, ATX-S10(Na). *Exp Eye Res.* 2001;72:13–22.
 151. Gohto Y, Obana A, Kanai M, Nagata S, Miki T, Nakajima S. Photodynamic therapy for corneal neovascularization using topically administered ATX-S10 (Na). *Ophthalmic Surg Lasers.* 2000;31:55–60.
 152. Glanzmann T, Hadjur C, Zellweger M, Grosjean P, Forrer M, Ballini JP, Monnier P, van den Bergh H, Lim CK, Wagnieres G. Pharmacokinetics of tetra(m-hydroxyphenyl)chlorin in human plasma and individualized light dosimetry in photodynamic therapy. *Photochem Photobiol.* 1998;67:596–602.
 153. Jones HJ, Vernon DI, Brown SB. Photodynamic therapy effect of m-THPC (Foscan) in vivo: correlation with pharmacokinetics. *Br J Cancer.* 2003;89:398–404.
 154. Chen B, Roskams T, de Witte PA. Antivascular tumor eradication by hypericin-mediated photodynamic therapy. *Photochem Photobiol.* 2002;76:509–13.
 155. Chen B, Roskams T, Xu Y, Agostinis P, de Witte PA. Photodynamic therapy with hypericin induces vascular damage and apoptosis in the RIF-1 mouse tumor model. *Int J Cancer.* 2002;98:284–90.
 156. Chen B, Xu Y, Roskams T, Delaey E, Agostinis P, Vandenheede JR, de Witte P. Efficacy of antitumoral photodynamic therapy with hypericin: relationship between biodistribution and photodynamic effects in the RIF-1 mouse tumor model. *Int J Cancer.* 2001;93:275–82.
 157. Chen B, Zupko I, de Witte PA. Photodynamic therapy with hypericin in a mouse P388 tumor model: vascular effects determine the efficacy. *Int J Oncol.* 2001;18:737–42.
 158. Dolmans DE, Fukumura D, Jain RK. Photodynamic therapy for cancer. *Nat Rev Cancer.* 2003;3:380–7.